



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>5</sup> :  C12N 1/21, 15/29, 15/82 C07H 15/12		A1	(11) International Publication Number: <b>WO 91/13972</b>  (43) International Publication Date: 19 September 1991 (19.09.91)													
<p>(21) International Application Number: PCT/US91/01746</p> <p>(22) International Filing Date: 14 March 1991 (14.03.91)</p> <p>(30) Priority data:</p> <table> <tr> <td>494,106</td> <td>16 March 1990 (16.03.90)</td> <td>US</td> </tr> <tr> <td>567,373</td> <td>13 August 1990 (13.08.90)</td> <td>US</td> </tr> <tr> <td>615,784</td> <td>14 November 1990 (14.11.90)</td> <td>US</td> </tr> </table> <p>(60) Parent Application or Grant</p> <p>(63) Related by Continuation</p> <table> <tr> <td>US</td> <td>494,106 (CIP)</td> </tr> <tr> <td>Filed on</td> <td>16 March 1990 (16.03.90)</td> </tr> </table> <p>(71) Applicant (for all designated States except US): CALGENE, INC. [US/US]; 1920 Fifth Street, Davis, CA 95616 (US).</p>		494,106	16 March 1990 (16.03.90)	US	567,373	13 August 1990 (13.08.90)	US	615,784	14 November 1990 (14.11.90)	US	US	494,106 (CIP)	Filed on	16 March 1990 (16.03.90)	<p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (for US only) : THOMPSON, Gregory, A. [US/US]; 5127 Cowell Blvd., Davis, CA 95616 (US). KNAUF, Vic, C. [US/US]; 1013 Hillview Lane, Winters, CA 95694 (US).</p> <p>(74) Agents: LASSEN, Elizabeth et al.; Calgene, Inc., 1920 Fifth Street, Davis, CA 95616 (US).</p> <p>(81) Designated States: AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US.</p> <p><b>Published</b>  <i>With international search report.</i>  <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>	
494,106	16 March 1990 (16.03.90)	US														
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(54) Title: PLANT DESATURASES - COMPOSITIONS AND USES

## (57) Abstract

By this invention, compositions and methods of use of plant desaturase enzymes, especially  $\Delta$ -9 desaturases, are provided. Of special interest are methods and compositions of amino acids and nucleic acid sequences related to biologically active plant desaturases as well as sequences, especially nucleic acid sequences, which are to be used as probes, vectors for transformation or cloning intermediates. Biologically active sequences may be found in a sense or anti-sense orientation as to transcriptional regulatory regions found in various constructs.

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PLANT DESATURASES -  
COMPOSITIONS AND USES

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This application is a continuation-in-part of USSN 07/494,106 filed on March 16, 1990 and a continuation-in-part of USSN 07/567,373 filed on August 13, 1990 and a continuation-in-part of USSN 07/615,784 filed on November 10 14, 1990.

Technical Field

The present invention is directed to desaturase enzymes relevant to fatty acid synthesis in plants, 15 enzymes, amino acid and nucleic acid sequences and methods related thereto, and novel plant entities and/or oils and methods related thereto.

INTRODUCTION

20 Background

Novel vegetable oils compositions and/or improved means to obtain or manipulate fatty acid compositions, from biosynthetic or natural plant sources, are needed. Depending upon the intended oil use, various different oil 25 compositions are desired. For example, edible oil sources containing the minimum possible amounts of saturated fatty acids are desired for dietary reasons and alternatives to current sources of highly saturated oil products, such as tropical oils, are also needed.

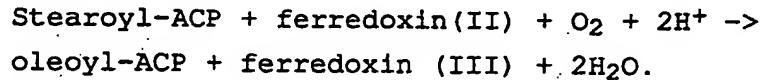
30 One means postulated to obtain such oils and/or modified fatty acid compositions is through the genetic engineering of plants. However, in order to genetically engineer plants one must have in place the means to transfer genetic material to the plant in a stable and 35 heritable manner. Additionally, one must have nucleic acid sequences capable of producing the desired phenotypic result, regulatory regions capable of directing the correct application of such sequences, and the like. Moreover, it

should be appreciated that to produce a desired modified oils phenotype requires that the Fatty Acid Synthetase (FAS) pathway of the plant is modified to the extent that the ratios of reactants are modulated or changed.

5        Higher plants appear to synthesize fatty acids via a common metabolic pathway in plant plastid organelles (i.e., chloroplasts, proplastids, or other related organelles) as part of the FAS complex. Outside of plastid organelles, fatty acids are incorporated into triglycerides and used in  
10      plant membranes and in neutral lipids. In developing seeds, where oils are produced and stored as sources of energy for future use, FAS occurs in proplastids.

15       The production of fatty acids begins in the plastid with the reaction between Acyl Carrier Protein (ACP) and acetyl-CoA to produce acetyl-ACP. Through a sequence of cyclical reactions, the acetyl-ACP is elongated to 16- and 18- carbon fatty acids. The longest chain fatty acids produced by the FAS are 18 carbons long. Monounsaturated fatty acids are also produced in the plastid through the  
20      action of a desaturase enzyme.

Common plant fatty acids, such as oleic, linoleic and  $\alpha$ -linolenic acids, are the result of sequential desaturation of stearate. The first desaturation step is the desaturation of stearoyl-ACP (C18:0) to form oleoyl-ACP  
25      (C18:1) in a reaction often catalyzed by a  $\Delta$ -9 desaturase, also often referred to as a "stearoyl-ACP desaturase" because of its high activity toward stearate the 18 carbon acyl-ACP. The desaturase enzyme functions to add a double bond at the ninth carbon in accordance with the following  
30      reaction (I):



$\Delta$ -9 desaturases have been studied in partially purified preparations from numerous plant species. Reports  
35      indicate that the protein is a dimer, perhaps a homodimer, displaying a molecular weight of 68 kD ( $\pm$ 8 kD) by gel-filtration and a molecular weight of 36 kD by SDS-polyacrylamide gel electrophoresis.

In subsequent sequential steps for triglyceride production, polyunsaturated fatty acids may be produced. These desaturations occur outside of the plastid as a result of the action of membrane-bound enzymes. Additional 5 double bonds are added at the twelve position carbon and thereafter, if added, at the 15 position carbon through the action of  $\Delta$ -12 desaturase and  $\Delta$ -15 desaturase, respectively.

Obtaining nucleic acid sequences capable of producing 10 a phenotypic result in FAS, desaturation and/or incorporation of fatty acids into a glycerol backbone to produce an oil is subject to various obstacles including but not limited to the identification of metabolic factors of interest, choice and characterization of a protein 15 source with useful kinetic properties, purification of the protein of interest to a level which will allow for its amino acid sequencing, utilizing amino acid sequence data to obtain a nucleic acid sequence capable of use as a probe to retrieve the desired DNA sequence, and the preparation 20 of constructs, transformation and analysis of the resulting plants.

Thus, the identification of enzyme targets and useful 25 plant sources for nucleic acid sequences of such enzyme targets capable of modifying fatty acid compositions are needed. Ideally, an enzyme target will be amenable to one or more applications alone or in combination with other nucleic acid sequences relating to increased/decreased oil production, the ratio of saturated to unsaturated fatty acids in the fatty acid pool, and/or to novel oils 30 compositions as a result of the modifications to the fatty acid pool. Once enzyme target(s) are identified and qualified, quantities of protein and purification protocols are needed for sequencing. Ultimately, useful nucleic acid constructs having the necessary elements to provide a 35 phenotypic modification and plants containing such constructs are needed.

Relevant Literature

A 200-fold purification of *Carthamus tinctorius* ("safflower") stearoyl-ACP desaturase was reported by McKeon & Stumpf in 1982, following the first publication of 5 their protocol in 1981. McKeon, T. & Stumpf, P. *J.Biol.Chem.* (1982) 257:12141-12147; McKeon, T. & Stumpf, P. *Methods in Enzymol.* (1981) 71:275-281.

BRIEF DESCRIPTION OF THE DRAWINGS

10 Fig. 1 provides amino acid sequence of fragments relating to *C. tinctorius* desaturase. Fragments F1 through F11 are also provided in the sequence listing as SEQ ID NO: 1 through SEQ ID NO: 11, respectively. Each fragment represents a synthesis of sequence information from 15 peptides originating from different digests which have been matched and aligned. In positions where there are two amino acids indicated, the top one corresponds to that found in the translation of the cDNA; the lower one was detected either as a second signal at the same position of 20 one of the sequenced peptides, or as a single unambiguous signal found in one or more of the overlapping peptides comprising the fragment. Residues in F9 shown in lower case letters represent positions where the called sequence does not agree with that predicted from the cDNA, but where 25 the amino acid assignment is tentative because of the presence of a contaminating peptide. The standard one letter code for amino acid residues has been used. X represents a position where no signal was detectable, and which could be a modified residue. F1 corresponds to the 30 N-terminal sequence of the mature protein. The underlined region in F2 is the sequence used in designing PCR primers for probe synthesis.

Fig. 2 provides a cDNA sequence (SEQ ID NO: 12) and the corresponding translational peptide sequence (SEQ ID 35 NO: 13) derived from *C. tinctorius* desaturase. The cDNA sequence includes both the plastid transit peptide encoding sequence and the sequence encoding the mature protein.

Fig. 3 provides cDNA sequence of *Ricinus communis* desaturase. Fig. 3A provides preliminary partial cDNA sequence of a 1.7 kb clone of *R. communis* desaturase (SEQ ID NO: 14). The sequence is from the 5' end of the clone.

5 Fig. 3B provides the complete cDNA sequence of the approximately 1.7 kb clone (SEQ ID NO: 15) and the corresponding translational peptide sequence (SEQ ID NO: 16).

Fig. 4 provides sequence of *Brassica campestris* desaturase. Fig. 4A represents partial DNA sequence of a 1.6 kb clone pCGN3235 (SEQ ID NO: 17), from the 5' end of the clone. Fig. 4B represents partial DNA sequence of a 1.2 kb clone, pCGN3236, from the 5' end of the clone (SEQ ID NO: 18). Initial sequence for the 3' ends of the two *B. campestris* desaturase clones indicates that pCGN3236 is a shorter cDNA for the same clone as pCGN3235. Fig. 4C provides complete cDNA sequence of *B. campestris* desaturase above, pCGN3235 (SEQ ID NO: 19) and the corresponding translational peptide sequence (SEQ ID NO: 20).

20 Fig. 5 provides preliminary partial cDNA sequence of *Simmondsia chinensis* desaturase (SEQ ID NO: 43). The translated amino acid sequence is also shown.

Fig. 6 shows the design of forward and reverse primers (SEQ ID NO: 21 through SEQ ID NO: 26) used in polymerase chain reaction (PCR) from the sequence of *C. tinctorius* desaturase peptide "Fragment F2" (SEQ ID NO: 2).

Fig. 7 provides maps of desaturase cDNA clones showing selected restriction enzyme sites. Fig. 7A represents a *C. tinctorius* clone, Fig. 7B represents a *R. communis* clone,

30 and Fig. 7C represents a *B. campestris* clone.

Fig. 8 provides approximately 3.4 kb of genomic sequence of Bce4 (SEQ ID NO: 27).

Fig. 9 provides approximately 4 kb of genomic sequence of Bcg 4-4 ACP sequence (SEQ ID NO: 28).

35 Fig. 10 provides a restriction map of cloned λCGN 1-2 showing the entire napin coding region sequence as well as extensive 5' upstream and 3' downstream sequences (SEQ ID NO: 29).

SUMMARY OF THE INVENTION

By this invention, compositions and methods of use of 5 plant desaturase enzymes, especially  $\Delta$ -9 desaturases, are provided. Of special interest are methods and compositions of amino acids and nucleic acid sequences related to biologically active plant desaturases as well as sequences, especially nucleic acid sequences, which are to be used as 10 probes, vectors for transformation or cloning intermediates. Biologically active sequences may be found in a sense or anti-sense orientation as to transcriptional regulatory regions found in various constructs.

A first aspect of this invention relates to *C. tinctorius*  $\Delta$ -9 desaturase substantially free of seed 15 storage protein. Amino acid sequence of this desaturase is provided in Fig. 2 and as SEQ ID NO: 13.

DNA sequence of *C. tinctorius* desaturase gene (SEQ ID NO: 12) is provided, as well as DNA sequences of desaturase 20 genes from a *Ricinus* (SEQ ID NO: 14 and SEQ ID NO: 15) a *Brassica* (SEQ ID NO: 17 through SEQ ID NO: 19) and a *Simmondsia* (SEQ ID NO: 43) plant.

In yet a different embodiment of this invention, plant 25 desaturase cDNA of at least 10 nucleotides or preferably at least 20 nucleotides and more preferably still at least 50 nucleotides, known or homologously related to known  $\Delta$ -9 desaturase(s) is also provided. The cDNA encoding precursor desaturase or, alternatively, biologically active, mature desaturase is provided herein.

30 Methods to use nucleic acid sequences to obtain other plant desaturases are also provided. Thus, a plant desaturase may be obtained by the steps of contacting a nucleic acid sequence probe comprising nucleotides of a known desaturase sequence and recovery of DNA sequences 35 encoding plant desaturase having hybridized with the probe.

This invention also relates to methods for obtaining plant  $\Delta$ -9 desaturase by contacting an antibody specific to a known desaturase, such as *C. tinctorius* stearoyl-ACP

desaturase, with a candidate plant stearoyl-ACP desaturase under conditions conducive to the formation of an antigen:antibody immunocomplex and the recovery of the candidate plant stearoyl-ACP desaturase which reacts thereto.

5 In a further aspect of this invention DNA constructs comprising a first DNA sequence encoding a plant desaturase and a second DNA sequence which is not naturally found joined to said plant desaturase are provided. This 10 invention also relates to the presence of such constructs in host cells, especially plant host cells. In yet a different aspect, this invention relates to transgenic host cells which have an expressed desaturase therein.

15 Constructs of this invention may contain, in the 5' to 3' direction of transcription, a transcription initiation control regulatory region capable of promoting transcription in a host cell and a DNA sequence encoding plant desaturase. Transcription initiation control 20 regulatory regions capable of expression in prokaryotic or eukaryotic host cells are provided. Most preferred are transcription initiation control regions capable of expression in plant cells, and more preferred are transcription and translation initiation regions 25 preferentially expressed in plant cells during the period of lipid accumulation. The DNA sequence encoding plant desaturase of this invention may be found in either the sense or anti-sense orientation to the transcription initiation control region.

30 Specific constructs, expression cassettes having in the 5' to 3' direction of transcription, a transcription and translation initiation control regulatory region comprising sequence immediately 5' to a structural gene 35 preferentially expressed in plant seed during lipid accumulation, a DNA sequence encoding desaturase, and sequence 3' to the structural gene are also provided. The construct may preferably contain DNA sequences encoding plant desaturase obtainable (included obtained) from *Carthamus*, *Rininus*, *Brassica* or *Simmondsia*  $\Delta$ -9 desaturase

genes. Transcription and translation initiation control regulatory regions are preferentially obtained from structural genes preferentially expressed in plant embryo tissue such as napin, seed-ACP or Bce-4.

5 By this invention, methods and constructs to inhibit the production of endogenous desaturase are also provided. For example, an anti-sense construct comprising, in the 5' to 3' direction of transcription, a transcription initiation control regulatory region functional in a plant 10 cell, and an anti-sense DNA sequence encoding a portion of a plant  $\Delta$ -9 desaturase may be integrated into a plant host cell to decrease desaturase levels.

In yet a different embodiment, this invention is directed to a method of producing plant desaturase in a 15 host cell comprising the steps of growing a host cell comprising an expression cassette, which would contain in the direction of transcription, a) a transcription and translation initiation region functional in said host cell, b) the DNA sequence encoding a plant desaturase in reading 20 frame with said initiation region, and c) and a transcript termination region functional in said host cell, under conditions which will promote the expression of the plant desaturase. Cells containing a plant desaturase as a 25 result of the production of the plant desaturase encoding sequence and also contemplated herein.

By this invention, a method of modifying fatty acid composition in a host plant cell from a given level of fatty acid saturation to a different level of fatty acid saturation is provided by growing a host plant cell having 30 integrated into its genome a recombinant DNA sequence encoding a plant desaturase in either a sense or anti-sense orientation under control of regulatory elements functional in said plant cell during lipid accumulation under conditions which will promote the activity of said 35 regulatory elements. Plant cells having such a modified level of fatty acid saturation are also contemplated hereunder. Oilseeds having such a modified level of fatty

acid saturation and oils produced from such oilseeds are further provided.

DETAILED DESCRIPTION OF THE INVENTION

5       A plant desaturase of this invention includes any sequence of amino acids, such as a protein, polypeptide, or peptide fragment, obtainable from a plant source which is capable of catalyzing the insertion of a first double bond into a fatty acyl-ACP moiety in a plant host cell, i.e., *in vivo*, or in a plant cell-like environment, i.e. *in vitro*. "A plant cell-like environment" means that any necessary conditions are available in an environment (i.e., such factors as temperatures, pH, lack of inhibiting substances) which will permit the enzyme to function. In particular, 10      this invention relates to enzymes which add such a first double bond at the ninth carbon position in a fatty acyl-ACP chain. There may be similar plant desaturase enzymes 15      of this invention with different specificities, such as the  $\Delta$ -12 desaturase of carrot.

20      Nucleotide sequences encoding desaturases may be obtained from natural sources or be partially or wholly artificially synthesized. They may directly correspond to a desaturase endogenous to a natural plant source or contain modified amino acid sequences, such as sequences 25      which have been mutated, truncated, increased or the like. Desaturases may be obtained by a variety of methods, including but not limited to, partial or homogenous purification of plant extracts, protein modeling, nucleic acid probes, antibody preparations and sequence 30      comparisons. Typically a plant desaturase will be derived in whole or in part from a natural plant source.

35      Of special interest are  $\Delta$ -9 desaturases which are obtainable, including those with are obtained, from *Carthamus*, *Ricinus*, *Simmondsia*, or *Brassica*, for example, *C. tinctorius*, *R. communis*, *S. chinensis* and *B. campestris*, respectively, or from plant desaturases which are obtainable through the use of these sequences. "Obtainable" refers to those desaturases which have

sufficiently similar sequences to that of the native sequences provided herein to provide a biologically active desaturase.

Once a DNA sequence which encodes a desaturase is obtained, it may be employed as a gene of interest in a nucleic acid construct or in probes in accordance with this invention. A desaturase may be produced in host cells for harvest or as a means of effecting a contact between the desaturase and its substrate. Constructs may be designed to produce desaturase in either prokaryotic or eukaryotic cells. Plant cells containing recombinant constructs encoding biologically active desaturase sequences, both expression and anti-sense constructs, as well as plants and cells containing modified levels of desaturase proteins are of special interest. For use in a plant cell, constructs may be designed which will effect an increase or a decrease in amount of endogenous desaturase available to a plant cell transformed with such a construct.

Where the target gene encodes an enzyme, such as a plant desaturase, which is already present in the host plant, there are inherent difficulties in analyzing mRNA, engineered protein or enzyme activity, and modified fatty acid composition or oil content in plant cells, especially in developing seeds; each of which can be evidence of biological activity. This is because the levels of the message, enzyme and various fatty acid species are changing rapidly during the stage where measurements are often made, and thus it can be difficult to discriminate between changes brought about by the presence of the foreign gene and those brought about by natural developmental changes in the seed. Where an expressed  $\Delta$ -9 desaturase DNA sequence is derived from a plant species heterologous to the plant host into which the sequence is introduced and has a distinguishable DNA sequence, it is often possible to specifically probe for expression of the foreign gene with oligonucleotides complimentary to unique sequences of the inserted DNA/RNA. And, if the foreign gene codes for a protein with slightly different protein sequence, it may be

possible to obtain antibodies which recognize unique epitopes on the engineered protein. Such antibodies can be obtained by mixing the antiserum to the foreign protein with extract from the host plant, or with extracts

5 containing the host plant enzyme. For example, one can isolate antibodies uniquely specific to a *C. tinctorius*  $\Delta$ -9 desaturase by mixing antiserum to the desaturase with an extract containing a *Brassica*  $\Delta$ -9 desaturase. Such an approach will allow the detection of *C. tinctorius*

10 desaturase in *Brassica* plants transformed with the *C. tinctorius* desaturase gene. In plants expressing an endogenous gene in an antisense orientation, the problem is slightly different. In this case, there are no specific reagents to measure expression of a foreign protein.

15 However, one is attempting to measure a decrease in an enzyme activity that normally is increasing during development. This makes detection of expression a simpler matter. In the final seed maturation phase, enzyme activities encoded by genes affecting oil composition

20 usually disappear and cannot be detected in final mature seed. Analysis of the fatty acid content may be performed by any manner known to those skilled in the art, including gas chromatography, for example.

By increasing the amount of desaturase available in

25 the plant cell, an increased percentage of unsaturated fatty acids may be provided; by decreasing the amount of desaturase, an increased percentage of saturated fatty acids may be provided. (Modifications in the pool of fatty acids available for incorporation into triglycerides may

30 likewise affect the composition of oils in the plant cell.) Thus, an increased expression of desaturase in a plant cell may result in increased proportion of fatty acids, such as one or more of palmitoleate (C16:1), oleate (C18:1), linoleate (C18:2) and linolenate (C18:3) are expected. Of

35 special interest is the production of triglycerides having increased levels of oleate. Using anti-sense technology, alternatively, a decrease in the amount of desaturase available to the plant cell is expected, resulting in a

higher percentage of saturates such as one or more of laurate (C12:0), myristate (C14:0), palmitate (C16:0), stearate (C18:0), arachidate (C20:0), behenate (C22:0) and lignocerate (C24:0). Of special interest is the production 5 of triglycerides having increased levels of stearate or palmitate and stearate. In addition, the production of a variety of ranges of such saturates is desired. Thus, plant cells having lower and higher levels of stearate fatty acids are contemplated. For example, fatty acid 10 compositions, including oils, having a 10% level of stearate as well as compositions designed to have up to a 60% level of stearate or other such modified fatty acid(s) composition are contemplated.

The modification of fatty acid compositions may also 15 affect the fluidity of plant membranes. Different lipid concentrations have been observed in cold-hardened plants, for example. By this invention, one may be capable of introducing traits which will lend to chill tolerance. Constitutive or temperature inducible transcription 20 initiation regulatory control regions may have special applications for such uses.

Other applications for use of cells or plants producing desaturase may also be found. For example, potential herbicidal agents selective for plant desaturase 25 may be obtained through screening to ultimately provide environmentally safe herbicide products. The plant desaturase can also be used in conjunction with chloroplast lysates to enhance the production and/or modify the composition of the fatty acids prepared *in vitro*. The desaturase can also be used for studying the mechanism of 30 fatty acid formation in plants and bacteria. For these applications, constitutive promoters may find the best use.

Constructs which contain elements to provide the transcription and translation of a nucleic acid sequence of 35 interest in a host cell are "expression cassettes". Depending upon the host, the regulatory regions will vary, including regions from structural genes from viruses, plasmid or chromosomal genes, or the like. For expression

in prokaryotic or eukaryotic microorganisms, particularly unicellular hosts, a wide variety of constitutive or regulatable promoters may be employed. Among transcriptional initiation regions which have been 5 described are regions from bacterial and yeast hosts, such as *E. coli*, *B. subtilis*, *Saccharomyces cerevisiae*, including genes such as  $\beta$ -galactosidase, T7 polymerase, *trp* E and the like.

A recombinant construct for expression of desaturase 10 in a plant cell ("expression cassette") will include, in the 5' to 3' direction of transcription, a transcription and translation initiation control regulatory region (the transcriptional and translational initiation regions together often also known as a "promoter") functional in a 15 plant cell, a nucleic acid sequence encoding a plant desaturase, and a transcription termination region. Numerous transcription initiation regions are available which provide for a wide variety of constitutive or regulatable, e.g., inducible, transcription of the 20 desaturase structural gene. Among transcriptional initiation regions used for plants are such regions associated with cauliflower mosaic viruses (35S, 19S), and structural genes such as for nopaline synthase or mannopine synthase or napin and ACP promoters, etc. The 25 transcription/translation initiation regions corresponding to such structural genes are found immediately 5' upstream to the respective start codons. Thus, depending upon the intended use, different promoters may be desired.

Of special interest in this invention are the use of 30 promoters which are capable of preferentially expressing the desaturase in seed tissue, in particular, at early stages of seed oil formation. Examples of such seed-specific promoters include the region immediately 5' upstream of napin or seed ACP genes, such as described in 35 co-pending USSN 147,781, and the Bce-4 gene such as described in co-pending USSN 494,722. Alternatively, the use of the 5' regulatory region associated with an endogenous plant desaturase structural gene and/or the

transcription termination regions found immediately 3' downstream to the gene, may often be desired.

In addition, for some applications, use of more than one promoter may be desired. For example, one may design a 5 dual promoter expression cassette each promoter having a desaturase sequence under its regulatory control. For example, the combination of an ACP and napin cassette could be useful for increased production of desaturase in a seed-specific fashion over a longer period of time than either 10 individually.

To decrease the amount of desaturase found in a plant host cell, anti-sense constructs may be prepared and then inserted into the plant cell. By "anti-sense" is meant a DNA sequence in the 5' to 3' direction of transcription in 15 relation to the transcription initiation region, which encodes a sequence complementary to the sequence of a native desaturase. It is preferred that an anti-sense plant desaturase sequence be complementary to a plant desaturase gene indigenous to the plant host. Sequences 20 found in an anti-sense orientation may be found in constructs providing for transcription or transcription and translation of the DNA sequence encoding the desaturase, including expression cassettes. Constructs having more than one desaturase sequence under the control of more than 25 one promoter or transcription initiation region may also be employed with desaturase constructs. Various transcription initiation regions may be employed. One of ordinary skill in the art can readily determine suitable regulatory regions. Care may be necessary in selecting transcription 30 initiation regions to avoid decreasing desaturase activity in plant cells other than oilseed tissues. Any transcription initiation region capable of directing expression in a plant host which causes initiation of adequate levels of transcription selectively in storage 35 tissues during seed development for example, should be sufficient. As such, seed specific promoters may be desired. Other manners of decreasing the amount of endogenous plant desaturase, such as ribozymes or the

screening of plant cells transformed with constructs for rare events containing sense sequences which in fact act to decrease desaturase expression, are also contemplated herein. Other analogous methods may be applied by those of ordinary skill in the art.

By careful selection of plants, transformants having particular oils profiles may be obtained. This may in part depend upon the qualities of the transcription initiation region(s) employed or may be a result of culling 10 transformation events to exploit the variabilities of expression observed.

In order to obtain the nucleic acid sequences encoding *C. tinctorius* desaturase, a protein preparation free of a major albumin-type contaminant is required. As 15 demonstrated more fully in the Examples, the protocols of McKeon and Stumpf, *supra*, result in a preparation contaminated with a seed storage protein. Removal of the protein contaminant may be effected by application of a reverse-phase HPLC, or alternatively, by application of a 20 reduction and alkylation step followed by electrophoresis and blotting, for example. Other purification methods may be employed as well, now that the presence of the contaminant is confirmed and various properties thereof described. Once the purified desaturase is obtained it may 25 be used to obtain the corresponding amino acid and/or nucleic acid sequences thereto in accordance with methods familiar to those skilled in the art. Approximately 90% of the total amino acid sequence of the *C. tinctorius* desaturase is provided in Fig. 1 and in SEQ ID NOS: 1-11. 30 The desaturase produced in accordance with the subject invention can be used in preparing antibodies for assays for detecting plant desaturase from other sources.

A nucleic acid sequence of this invention may include genomic or cDNA sequence and mRNA. A cDNA sequence may or 35 may not contain pre-processing sequences, such as transit peptide sequences. Transit peptide sequences facilitate the delivery of the protein to a given organelle and are

cleaved from the amino acid moiety upon entry into the organelle, releasing the "mature" sequence.

In Fig. 2 and SEQ ID NO: 13, the sequence of the *C. tinctorius* desaturase precursor protein is provided; both 5 the transit peptide and mature protein sequence are shown. Also provided in this invention are cDNA sequences relating to *R. communis* desaturase (Fig. 3 and SEQ ID NOS: 14-15), *B. campestris* desaturase (Fig. 4 and SEQ ID NOS: 17-19) and *S. chinesis* (Fig. 5 and SEQ ID NOS: 43).

10 The use of the precursor cDNA sequence is preferred in desaturase expression cassettes. In addition, desaturase transit peptide sequences may be employed to translocate other proteins of interest to plastid organelles for a variety of uses, including the modulation of other enzymes 15 related to the FAS pathway. See, European Patent Application Publication No. 189,707.

20 As described in more detail below, the complete genomic sequence of a desaturase may be obtained by the screening of a genomic library with a desaturase cDNA probe and isolating those sequences which regulate expression in seed tissue. In this manner, the transcription, 25 translation initiation regions and/or transcript termination regions of the desaturase may be obtained for use in a variety of DNA constructs, with or without the respective desaturase structural gene.

30 Other nucleic acid sequences "homologous" or "related" to DNA sequences encoding other desaturases are also provided. "Homologous" or "related" includes those nucleic acid sequences which are identical or conservatively substituted as compared to the exemplified *C. tinctorius*, *R. communis*, *S. chinesis* or *B. campestris* desaturase sequences of this invention or a plant desaturase which has in turn been obtained from a plant desaturase of this invention. By conservatively substituted is meant that 35 codon substitutions encode the same amino acid, as a result of the degeneracy of the DNA code, or that a different amino acid having similar properties to the original amino acid is substituted. One skilled in the art will readily

recognize that antibody preparations, nucleic acid probes (DNA and RNA) sequences encoding and the like may be prepared and used to screen and recover desaturase from other plant sources. Typically, nucleic acid probes are 5 labeled to allow detection, preferably with radioactivity although enzymes or other methods may also be used. For immunological screening methods, antibody preparations either monoclonal or polyclonal are utilized. Polyclonal antibodies, although less specific, typically are more 10 useful in gene isolation. For detection, the antibody is labeled using radioactivity or any one of a variety of second antibody/enzyme conjugate systems that are commercially available. Examples of some of the available antibody detection systems are described by Oberfilder 15 (*Focus* (1989) BRL Life Technologies, Inc., 11:1-5).

A "homologous" or "related" nucleic acid sequence will show at least about 60% homology, and more preferably at least about 70% homology, between the known desaturase sequence and the desired candidate plant desaturase of 20 interest, excluding any deletions which may be present. Homology is determined upon comparison of sequence information, nucleic acid or amino acid, or through hybridization reactions. Amino acid sequences are considered homologous by as little as 25% sequence identity 25 between the two complete mature proteins. (See generally, Doolittle, R.F., of URFS and ORFS, University Science Books, CA, 1986.)

Oligonucleotide probes can be considerably shorter than the entire sequence, but should be at least about 10, 30 preferably at least about 15, more preferably at least 20 nucleotides in length. When shorter length regions are used for comparison, a higher degree of sequence identity is required than for longer sequences. Shorter probes are often particularly useful for polymerase chain reactions 35 (PCR), especially when highly conserved sequences can be identified. (See, Gould, et al., *PNAS USA* (1989) 86:1934-1938.) Longer oligonucleotides are also useful, up to the full length of the gene encoding the polypeptide of

interest. When longer nucleic acid fragments are employed (>100 bp) as probes, especially when using complete or large cDNA sequences, one would screen with low stringencies (for example 40-50°C below the melting 5 temperature of the probe) in order to obtain signal from the target sample with 20-50% deviation, i.e., homologous sequences. (See, Beltz, et al., *Methods in Enzymology* (1983) 100:266-285.) Both DNA and RNA probes can be used.

A genomic library prepared from the plant source of 10 interest may be probed with conserved sequences from a known desaturase to identify homologously related sequences. Use of the entire cDNA may be employed if shorter probe sequences are not identified. Positive clones are then analyzed by restriction enzyme digestion 15 and/or sequencing. When a genomic library is used, one or more sequences may be identified providing both the coding region, as well as the transcriptional regulatory elements of the desaturase gene from such plant source. In this general manner, one or more sequences may be identified 20 providing both the coding region, as well as the transcriptional regulatory elements of the desaturase gene from such plant source.

In use, probes are typically labeled in a detectable manner (for example with  $^{32}\text{P}$ -labeled or biotinylated 25 nucleotides) and are incubated with single-stranded DNA or RNA from the plant source in which the gene is sought, although unlabeled oligonucleotides are also useful. Hybridization is detected by means of the label after single-stranded and double-stranded (hybridized) DNA or 30 DNA/RNA have been separated, typically using nitrocellulose paper or nylon membranes. Hybridization techniques suitable for use with oligonucleotides are well known to those skilled in the art. Thus, plant desaturase genes may be isolated by various techniques from any convenient plant. 35 Plant desaturase of developing seed obtained from other oilseed plants, such as soybean, coconut, oilseed rape, sunflower, oil palm, peanut, cocoa, cotton, corn and the like are desired as well as from non-traditional oil

sources, including but not limited to spinach chloroplast, avocado mesocarp, cuphea, California Bay, cucumber, carrot, meadowfoam, *Oenothera* and *Euglena gracillisis*.

Once the desired plant desaturase sequence is  
5 obtained, it may be manipulated in a variety of ways. Where the sequence involves non-coding flanking regions, the flanking regions may be subjected to resection, mutagenesis, etc. Thus, transitions, transversions, deletions, and insertions may be performed on the naturally  
10 occurring sequence. In addition, all or part of the sequence may be synthesized, where one or more codons may be modified to provide for a modified amino acid sequence, or one or more codon mutations may be introduced to provide for a convenient restriction site or other purpose involved  
15 with construction or expression. The structural gene may be further modified by employing synthetic adapters, linkers to introduce one or more convenient restriction sites, or the like.

Recombinant constructs containing a nucleic acid  
20 sequence encoding a desaturase of this invention may be combined with other, i.e. "heterologous," DNA sequences in a variety of ways. By heterologous DNA sequences is meant any DNA sequence which is not naturally found joined to the native desaturase, including combinations of DNA sequences  
25 from the same plant of the plant desaturase which are not naturally found joined together. In a preferred embodiment, the DNA sequence encoding a plant desaturase is combined in a DNA construct having, in the 5' to 3' direction of transcription, a transcription initiation  
30 control region capable of promoting transcription in a host cell, and a DNA sequence encoding a desaturase in either a sense or anti-sense orientation. As described in more detail elsewhere, a variety of regulatory control regions containing transcriptional or transcriptional and  
35 translational regions may be employed, including all or part of the non-coding regions of the plant desaturase.

The open reading frame coding for the plant desaturase or functional fragment thereof will be joined at its 5' end

to a transcription initiation regulatory control region. In some instances, such as modulation of plant desaturase via a desaturase in an anti-sense orientation, a transcription initiation region or transcription/ 5 translation initiation region may be used. In embodiments wherein the expression of the desaturase protein is desired in a plant host, a transcription/ translation initiation regulatory region, is needed. Additionally, modified promoters, i.e., having transcription initiation regions 10 derived from one gene source and translation initiation regions derived from a different gene source or enhanced promoters, such as double 35S CaMV promoters, may be employed for some applications.

As described above, of particular interest are those 15 5' upstream non-coding regions which are obtained from genes regulated during seed maturation, particularly those preferentially expressed in plant embryo tissue, such as ACP-and napin-derived transcription initiation control regions. Such regulatory regions are active during lipid 20 accumulation and therefore offer potential for greater control and/or effectiveness to modify the production of plant desaturase and/or modification of the fatty acid composition. Especially of interest are transcription initiation regions which are preferentially expressed in 25 seed tissue, i.e., which are undetectable in other plant parts. For this purpose, the transcript initiation region of acyl carrier protein isolated from *B. campestris* seed and designated as "Bcg 4-4" and an unidentified gene isolated from *B. campestris* seed and designated as "Bce-4" 30 are also of substantial interest.

Briefly, Bce4 is found in immature embryo tissue at least as early as 11 days after anthesis (flowering), peaking about 6 to 8 days later or 17-19 days post-anthesis, and becoming undetectable by 35 days post- 35 anthesis. The timing of expression of the Bce4 gene closely follows that of lipid accumulation in seed tissue. Bce4 is primarily detected in seed embryo tissue and to a lesser extent found in the seed coat. Bce4 has not been

detected in other plant tissues tested, root, stem and leaves.

Approximately 3.4 kb genomic sequence of Bce4 is provided in Fig. 8 and as SEQ ID NO: 27, including about 1 kb 5' to the structural gene, about 0.3 kb of the Bce4 coding gene sequence, and about 2.1 kb of the non-coding regulatory 3' sequence. Bce4 transcript initiation regions will contain at least 1 kb and more preferably about 5 to about 7.5 kb of sequence immediately 5' to the Bce4 structural gene.

The Bcg 4-4 ACP message presents a similar expression profile to that of Bce4 and, therefore, also corresponds to lipid accumulation in the seed tissue. Bcg 4-4 is not found in the seed coat and may show some differences in expression level, as compared to Bce4, when the Bcg 4-4 5' non-coding sequence is used to regulate transcription or transcription and translation of a plant stearoyl-ACP desaturase of this invention. Genomic sequence of Bcg 4-4 is provided in Fig. 9 and as SEQ ID NO: 28, including about 1.5 kb 5' to the structural gene, about 1.2 kb of the Bcg 4-4 (ACP) structural gene sequence, and about 1.3 kb of the non-coding regulatory 3' sequence.

The napin 1-2 message is found in early seed development and thus, also offers regulatory regions which can offer preferential transcriptional regulation of a desired DNA sequence of interest such as the plant desaturase DNA sequence of this invention during lipid accumulation. Napins are one of the two classes of storage proteins synthesized in developing *Brassica* embryos (Bhatty, et al., *Can J. Biochem.* (1968) 46:1191-1197) and have been used to direct tissue-specific expression when reintroduced into the *Brassica* genome (Radke, et al., *Theor. Appl. Genet.* (1988) 75:685-694). Genomic sequence of napin 1-2 is provided in Fig. 10 and as SEQ ID NO: 29, including about 1.7 kb 5' to the structural gene and about 1.3 kb of the non-coding regulatory 3' sequence.

Regulatory transcript termination regions may be provided in DNA constructs of this invention as well.

Transcript termination regions may be provided by the DNA sequence encoding the plant desaturase or a convenient transcription termination region derived from a different gene source, especially the transcript termination region 5 which is naturally associated with the transcript initiation region. The transcript termination region will contain at least about 1 kb, preferably about 3 kb of sequence 3' to the structural gene from which the termination region is derived.

10 In developing the DNA construct, the various components of the construct or fragments thereof will normally be inserted into a convenient cloning vector which is capable of replication in a bacterial host, e.g., *E. coli*. Numerous vectors exist that have been described in 15 the literature. After each cloning, the plasmid may be isolated and subjected to further manipulation, such as restriction, insertion of new fragments, ligation, deletion, insertion, resection, etc., so as to tailor the components of the desired sequence. Once the construct has 20 been completed, it may then be transferred to an appropriate vector for further manipulation in accordance with the manner of transformation of the host cell.

Normally, included with the DNA construct will be a structural gene having the necessary regulatory regions for 25 expression in a host and providing for selection of transformant cells. The gene may provide for resistance to a cytotoxic agent, e.g. antibiotic, heavy metal, toxin, etc., complementation providing prototrophy to an auxotrophic host, viral immunity or the like. Depending 30 upon the number of different host species into which the expression construct or components thereof are introduced, one or more markers may be employed, where different conditions for selection are used for the different hosts.

The manner in which the DNA construct is introduced 35 into the plant host is not critical to this invention. Any method which provides for efficient transformation may be employed. Various methods for plant cell transformation include the use of Ti- or Ri-plasmids, microinjection,

electroporation, liposome fusion, DNA bombardment or the like. In many instances, it will be desirable to have the construct bordered on one or both sides by T-DNA, particularly having the left and right borders, more 5 particularly the right border. This is particularly useful when the construct uses *A. tumefaciens* or *A. rhizogenes* as a mode for transformation, although the T-DNA borders may find use with other modes of transformation.

Where *Agrobacterium* is used for plant cell 10 transformation, a vector may be used which may be introduced into the *Agrobacterium* host for homologous recombination with T-DNA or the Ti- or Ri-plasmid present in the *Agrobacterium* host. The Ti- or Ri-plasmid containing the T-DNA for recombination may be armed 15 (capable of causing gall formation) or disarmed (incapable of causing gall formation), the latter being permissible, so long as the *vir* genes are present in the transformed *Agrobacterium* host. The armed plasmid can give a mixture of normal plant cell and gall.

20 A preferred method for the use of *Agrobacterium* as the vehicle for transformation of plant cells employs a vector having a broad host range replication system, at least one T-DNA boundary and the DNA sequence or sequences of interest. Commonly used vectors include pRK2 or 25 derivatives thereof. See, for example, Ditta et al., *PNAS USA*, (1980) 77:7347-7351 and EPA 0 120 515, which are incorporated herein by reference. Normally, the vector will be free of genes coding for opines, oncogenes and *vir*-genes. Included with the expression construct and the T- 30 DNA will be one or more markers, which allow for selection of transformed *Agrobacterium* and transformed plant cells. A number of markers have been developed for use with plant cells, such as resistance to chloramphenicol, the aminoglycoside G418, hygromycin, or the like. The 35 particular marker employed is not essential to this invention, one or another marker being preferred depending on the particular host and the manner of construction.

The expression constructs may be employed with a wide variety of plant life, particularly plant life involved in the production of vegetable oils. These plants include, but are not limited to rapeseed, sunflower, *C. tinctorius*, 5 cotton, *Cuphea*, peanut, soybean, oil palm and corn. Anti-sense constructs may be employed in such plants which share complementarity between the endogenous sequence and the anti-sense desaturase. Of special interest is the use of an anti-sense construct having a *B. campestris* desaturase 10 in rapeseed, including *B. campestris* and *B. napus*.

For transformation of plant cells using *Agrobacterium*, explants may be combined and incubated with the transformed *Agrobacterium* for sufficient time for transformation, the bacteria killed, and the plant cells cultured in an 15 appropriate selective medium. Once callus forms, shoot formation can be encouraged by employing the appropriate plant hormones in accordance with known methods and the shoots transferred to rooting medium for regeneration of plants. The plants may then be grown to seed and the seed 20 used to establish repetitive generations and for isolation of vegetable oils compositions. A variety of stable genetic lines having fixed levels of saturation may be obtained and integrated into a traditional breeding program. Hemizygous and heterozygous lines or homozygous 25 lines may demonstrate different useful properties for oil production and/or breeding. For example, saturation levels may be increased up to 2-fold by the development of homozygous plants as compared with heterozygous (including hemizygous) plants.

30 The invention now being generally described, it will be more readily understood by reference to the following examples which are included for purposes of illustration only and are not intended to limit the present invention.

35

#### EXAMPLES

#### MATERIALS

Commercially available biological chemicals and chromatographic materials, including BSA, catalase (twice

crystallized from bovine liver), spinach ferredoxin, ferredoxin-NADP<sup>+</sup> oxidoreductase (spinach leaf), NADPH, unlabeled fatty acids, DEAE-cellulose (Whatman DE-52) CNBr-activated Sepharose 4B, and octyl-Sepharose, and Reactive Blue Agarose are from Sigma (St. Louis, MO). Triethylamine, trichloroacetic acid, guanidine-HCl, and hydrazine-hydrate are also from Sigma. Proteolytic enzymes, including endoproteinases lysC, gluC, and aspN are sequencing grade enzymes obtained from Boehringer Mannheim (Indianapolis, IN). Organic solvents, including acetone, acetonitrile, methanol, ether and petroleum ether are purchased from J.T. Baker (Phillipsburg, NJ); concentrated acids and sodium sulfate are also from J.T. Baker (Phillipsburg, NJ). HPLC grade acetonitrile and trifluoracetic acid (TFA) are obtained from Burdick and Jackson (Muskegon, MI), and from Applied Biosystems (Foster City, CA), respectively. Radiochemicals, including [9,10(n)-<sup>3</sup>H] oleic acid (10mCi/ $\mu$ mol) and [<sup>3</sup>H]-iodoacetic acid (64Ci/mol) are from New England Nuclear (Boston, MA). Phenacyl-8 Reagent (bromoacetophenone with a crown ether catalyst) used to prepare phenacyl esters of the fatty acids for analysis are from Pierce (Rockford, IL). C18 reversed-phase thin-layer chromatography plates are from Whatman (Clifton, NJ).

Acyl carrier protein (ACP) and acyl-ACP synthase are isolated from *E. coli* strain K-12 as described by Rock and Cronan (Rock and Cronan, *Methods in Enzymol* (1981) 71:341-351 and Rock et al., *Methods in Enzymol.* (1981) 72:397-403). The *E. coli* is obtainable from Grain Processing (Iowa) as frozen late-logarithmic phase cells.

[9,10(n)-<sup>3</sup>H]stearic acid is synthesized by reduction of [9,10(n)-<sup>3</sup>H]oleic acid with hydrazine hydrate essentially as described by Johnson and Gurr (*Lipids* (1971) 6:78-84). [9,10(n)-<sup>3</sup>H]oleic acid (2 mCi), supplemented with 5.58mg unlabeled oleic acid to give a final specific radioactivity of 100mCi/mmol, is dissolved in 2ml of acetonitrile, acidified with 40 $\mu$ l of glacial acetic acid, and heated to 55°C. Reduction is initiated with 100 $\mu$ l of

60% (w/w) hydrazine hydrate; oxygen is bubbled through the mixture continuously. After each hour acetonitrile is added to bring the volume back to 2ml and an additional 100 $\mu$ l of hydrazine hydrate is added. At the end of 5 hr. 5 the reaction is stopped by addition of 3ml of 2M HCl. The reaction products are extracted with three 3ml aliquots of petroleum ether and the combined ether extracts are washed with water, dried over sodium sulfate and evaporated to dryness. The dried reaction products are redissolved in 10 1.0ml acetonitrile and stored at -20°C. The distribution of fatty acid products in a 15 $\mu$ l aliquot is determined by preparation of phenacyl esters, which are then analyzed by thin layer chromatography on C-18 reverse phase plates developed with methanol:water:95:5 (v/v). Usually 15 reduction to [9,10(n)- $^3$ H]stearic acid is greater than 90%, a small amount of unreacted oleic acid may remain. The analysis is used to establish fraction of the total radioactivity that is present as stearate, and thereby to determine the exact substrate concentration in the enzyme 20 assay.

Acyl-ACP substrates, including [9,10(n)- $^3$ H] stearoyl-ACP are prepared and purified by the enzymatic synthesis procedure of Rock, Garwin, and Cronan (*Methods in Enzymol.* (1981) 72:397-403).

25 Acyl carrier protein was covalently bound to Sepharose 4B by reaction of highly purified ACP with CNBr-activated Sepharose 4B as described by McKeon and Stumpf (*J. Biol. Chem.* (1982) 257:12141-12147).

30 Example 1

In this example, an initial purification of *C. tinctorius* (safflower) desaturase, following the method of McKeon and Stumpf (*J. Biol. Chem.* (1982) 257:12141-12142), is described.

35 Assay: In each of the following steps, the presence of the enzyme is detected radiometrically by measuring enzyme-catalyzed release of tritium from [9,10(n)-

$^{3}\text{H}$ ]stearoyl-ACP. Preparation of this substrate is described in "Materials" above.

The assay is performed by mixing 150 $\mu\text{l}$  water, 5ml dithiothreitol (100mM, freshly prepared in water), 10 $\mu\text{l}$  5 bovine serum albumin (10mg/ml in water), 15 $\mu\text{l}$  NADPH (25mM, freshly prepared in 0.1M Tricine-HCl, pH 8.2), 25 $\mu\text{l}$  spinach ferredoxin (2mg/ml Sigma Type III in water), 3 $\mu\text{l}$  NADPH:ferredoxin oxidoreductase (2.5 units/ml from Sigma), and 1  $\mu\text{l}$  bovine liver catalase (800,000 units/ml from 10 Sigma); after 10 min at room temperature, this mixture is added to a 13x100 mm screw-cap test tube containing 250 $\mu\text{l}$  sodium 1,4-piperazinediethanesulfonate (0.1M, pH 6.0). Finally, 10 $\mu\text{l}$  of the sample to be assayed is added and the 15 reaction is started by adding 30 $\mu\text{l}$  of the substrate, [9,10(n)- $^{3}\text{H}$ ]stearoyl-ACP (100 $\mu\text{Ci}/\mu\text{mol}$ , 10 $\mu\text{M}$  in 0.1M sodium 1,4-piperazinediethanesulfonate, pH 5.8). After sealing with a cap, the reaction is allowed to proceed for 10 min. while shaking at 23°C. The reaction is terminated by 20 addition of 1.2ml of 5.8% tricholoracetic acid and the resulting precipitated acyl-ACP's are removed by centrifugation. The tritium released into the aqueous supernatant by the desaturase reaction is measured by 25 liquid scintillation spectrometry. One unit of activity is defined as the amount of enzyme required to convert 1 $\mu\text{mol}$  of stearoyl-ACP to oleoyl-ACP, or to release 4 $\mu\text{g}$ -atoms of  $^{3}\text{H}$  per minute.

*Source tissue:* Developing *C. tinctorius* seeds from greenhouse grown plants are harvested between 16 and 18 days after flowering, frozen in liquid nitrogen and stored 30 at -70°C until extracted.

*Acetone Powder:* Approximately 50g of frozen seeds are ground in liquid nitrogen and sieved to remove large seed coat pieces to provide a fine embryo powder. The powder is washed with acetone on a Buchner funnel until all 35 yellow color is absent from the filtrate. The powder is then air dried and further processed as described below, or may be stored frozen for at least a year at -70°C without loss of enzyme activity.

5        *Acetone Powder Extract:* The dried acetone powder is weighed and triturated with ten times its weight of 20mM potassium phosphate, pH 6.8; the mixture is then centrifuged at 12,000 x g for 20 minutes and decanted 5 through a layer of Miracloth (Calbiochem, La Jolla, CA).

10      *Ion Exchange Chromatography:* The acetone powder extract is then applied to a DEAE-cellulose column (Whatman DE-52) (1.5 x 12 cm) equilibrated with 20mM potassium phosphate, pH 6.8. The pass-through and a wash with one 10 column-volume (20ml) of buffer are pooled.

15      *Affinity Chromatography:* An affinity matrix for purification of the desaturase is prepared by reacting highly purified *E. coli* ACP, with CNBr-activated Sepharose 4B (Sigma). ACP (120mg) is reduced by treatment with 1mM 15 dithiothreitol for 30 min on ice, and then desalted on Sephadex G-10 (Pharmacia) equilibrated with 0.1M sodium bicarbonate, pH 6.0. The treated ACP (20 ml, 6 mg/ml) is then mixed with 20ml of CNBr-activated Sepharose 4B swollen in 0.1M sodium bicarbonate, pH 7.0, and the mixture is 20 allowed to stand at 4°C for one day. The gel suspension is then centrifuged, washed once with 0.1M sodium bicarbonate, pH 7.0, and then treated with 40ml 0.1M glycine, pH 8.0, for 4 hours at room temperature to block unreacted sites. The gel is then washed for five cycles with alternating 25 50ml volumes of 0.5M NaCl in 0.1M sodium acetate, pH 4.0, and 0.5M NaCl in 0.1M sodium bicarbonate, pH 6.5, to remove non-covalently bound ligand. The gel is loaded into a column (1.5 x 11.2 cm) and equilibrated in 20mM potassium phosphate, pH 6.8.

30      The combined fractions from the DE-52 column are applied to the column, which is subsequently washed with one column volume (20ml) of the equilibration buffer, and then with 2.5 column volumes (50ml) of 300mM potassium phosphate, pH 6.8. Fractions are assayed for protein using 35 the BCA Protein Assay Reagent (Pierce, Rockford, IL) to make sure that all extraneous protein has been eluted. Active Δ-9 desaturase is eluted from the column with 600mM potassium phosphate, pH 6.8. Active fractions are analyzed

by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE) on 0.75mm thick 8 x 12 cm mini-gels according to the method of Laemmli (*Nature* (1970) 227:680). The running gel contains 10% acrylamide in a 30/0.8 ratio of acrylamide to cross-linker bis-acrylamide. Those fractions containing a predominant band at approximately 43 kD are pooled and stored frozen at -70°C until final purification. The yield from 50g of seed tissue is approximately 60 $\mu$ g of protein as measured by amino acid analysis.

Further purification as described in Example 2 or Example 3 is then applied to the fractions pooled from the ACP-Sepharose column separation.

15 **Example 2**

In this example, a protocol for the final purification of *C. tinctorius* desaturase is described. Seeds are treated in accordance with Example 1.

Reverse-Phase HPLC: Fractions from the ACP-Sepharose column are pooled and applied to a Vydac C4 reverse-phase column (0.45 x 15 cm) equilibrated in 0.1% TFA, 7% acetonitrile. After a 10 min wash with 0.1% TFA, the column is eluted with a gradient of increasing acetonitrile (7%-70% v/v) in 0.1% TFA over a period of 45 min. The flow rate is 0.5ml/min throughout. Eluting components are monitored by absorbance at 214 nm.  $\Delta$ -9 desaturase elutes at about 42 min. (approximately 50% acetonitrile); the major contaminant protein remaining from ACP-affinity chromatography elutes at about 28 min. (approximately 30% acetonitrile). The substantially homogeneous desaturase, which is no longer active, is identified by SDS-PAGE, in which it exhibits a single band corresponding to a molecular weight of approximately 43 kD. The quantity of desaturase protein in the sample may be determined by amino acid analysis.

**Example 3**

In this example, a protocol for the final purification of *C. tinctorius* desaturase is described. Seeds are treated in accordance with Example 1.

5        *Reduction and Alkylation:* Protein is precipitated out of the pooled fraction solutions recovered from the ACP-Sepharose column with 10% (w/v) trichloroacetic acid, washed with cold (-20°C) acetone, and resuspended in 1 ml 500mM Tris-HCl, pH 8.6, containing 6M guanidine-HCl, 10mM 10 EDTA, and 3.2 mM dithiothreitol. After 2 hours, 3.52 µmol 15 [<sup>3</sup>H]-iodoacetic acid (64µCi/µmol, New England Nuclear) is added, and the reaction is allowed to proceed at room temperature in the dark for 2 hours, at which time the reaction is terminated by addition of 1µl (15µmol) B- 15 mercaptoethanol. The sample is then re-precipitated with 10% (w/v) trichloroacetic acid, and the pellet again washed with cold (-20°C) acetone and resuspended in Laemmli's SDS-sample buffer (*Nature* (1970) 227:680).

20        *SDS-Polyacrylamide Gel Electrophoresis:* The resulting sample is boiled for 5 min. and then applied to a 1.5 mm thick, 8 x 12 cm, SDS-polyacrylamide mini-gel prepared as described by Laemmli, *supra*. The running gel contains 17.5% acrylamide in a 30:0.13 ratio of acrylamide to cross-linking bis-acrylamide. Separation is achieved by 25 electrophoresis at 15 mA, for 2 hours at 4°C.

20        *Blotting from SDS-gels to PVDF Membrane:* Proteins are recovered from the gel by electroblotting at 5 mA/cm<sup>2</sup> to a four-layer sandwich of polyvinylidenedifluoride (PVDF) membrane for 2 h at 4°C in a buffer containing 10mM CAPS ("3-[cyclohexylamino]-1-propane-sulfonic acid"), pH 11. The membranes must be wetted in 50% methanol, prior to exposure to the blotting buffer. After blotting, the membrane layers are stained for 1-2 min. in 0.02% Coomassie Blue in 50% methanol, and then destained in 50% methanol. 35 The desaturase is identified as a band corresponding to a molecular weight of about 43 kD; the major contaminant runs at or near the dye front of the gel corresponding to a molecular weight less than 20 kD.

The desaturase band on the PVDF membrane may be applied directly to the Edman sequencer (Applied Biosystems Model 477A) for determination of the N-terminal sequence of the intact protein, or for more extensive sequence determination, may be eluted from the membrane in 40% acetonitrile to recover pure desaturase in solution. Acetonitrile is removed from the eluted desaturase by evaporation on a Speed-Vac (Savant; Farmingdale, NY), and the substantially homogeneous Δ-9 desaturase is resuspended in an appropriate buffer for subsequent proteolytic digestion as described in Example 4. The quantity of desaturase protein present may be determined by amino acid analysis.

Alternatively, if the sample is to be digested with trypsin or gluC protease to generate peptides for amino acid sequence analysis, proteins may be electroblotted to nitrocellulose membranes and stained with Ponceau or amido black.

20 **Example 4**

In this example, a method for the determination of the amino acid sequence of a desaturase is described.

*Reduction and Alkylation:* Substantially homogenous stearoyl-ACP desaturase (See, Example 2) is reduced and alkylated with [<sup>3</sup>H]-iodacetic acid (See, Example 3), except that the final acetone-washed pellet is resuspended in the appropriate buffer for subsequent proteolysis. Reduction and alkylation assures complete denaturation of the protein so that complete proteolysis can occur. The sample may be alkylated with radiolabeled iodoacetamide or with 4-vinylpyridine instead of [<sup>3</sup>H]-iodacetic acid in substantially the same manner. Use of iodoacetic acid affords an alkylated sample with greater solubility, which is advantageous in subsequent sample manipulation.

35 *Proteolysis:* Substantially pure alkylated samples are digested with endoproteinase lysC. The sample is resuspended in 100 μl of 25 mM Tris-HCl, pH 8.8, containing 1 mM EDTA. Endoproteinase lysC is added to the sample in a

protease/desaturase ratio of 1/50 (w/w). Digestion is allowed to proceed at room temperature for 8 hours, at which time another equal amount of protease is added. After 18 more hours, 1  $\mu$ l of concentrated HCl is added to 5 stop proteolysis, and the sample is applied directly to a Vydac C18 reverse-phase column (0.2 x 15 cm) equilibrated in 7% acetonitrile (v/v) in 0.1 mM sodium phosphate, pH 2.2. After washing for 20 min with the equilibration buffer, peptides are eluted with a gradient in acetonitrile 10 (7-70%, v/v) over 120 min. Flow rate is 50  $\mu$ l/min, throughout. Eluting components are monitored by absorbance at 214 nm, and individual peptide peaks are collected as separate fractions. The peptide fractions are further purified by application to a second Vydac C18 reverse-phase 15 column (0.2 x 15 cm) equilibrated in 7% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid. Again, after a 20 min wash with equilibration buffer, the substantially pure peptides are eluted with a gradient (7-70%, v/v) of acetonitrile in 0.1% trifluoroacetic acid over 120 min. 20 The flow rate is 50  $\mu$ l/min, throughout. Eluting components are monitored by absorbance at 214 nm, and individual peptide peaks are collected as separate fractions. These substantially pure peptides are applied directly to the Edman sequencer (Applied Biosystems, Model 477A) for amino 25 acid sequence analysis. Alternatively, peptide fraction from the first HPLC purification in phosphate buffer, or from a single chromatography step in trifluoroacetic acid buffer, may be applied directly to the sequencer, but these fractions, in many cases, are not substantially pure and 30 yield mixed or ambiguous sequence information.

Other proteases may be used to digest desaturase, including but not limited to trypsin, gluC, and aspN. While the individual digest buffer conditions may be different, the protocols for digestion, peptide separation, 35 purification, and sequencing are substantially the same as those outlined for the digestion with lysC. Alternatively, desaturase may be digested chemically using cyanogen bromide (Gross *Methods Enzymol* (1967) 11:238-255 or Gross

and Witkop *J. Am. Chem. Soc.* (1961) 83:1510), hydroxylamine (Bornstein and Balian *Methods Enzymol.* (1977) 47:132-745), iodosobenzoic acid (Inglis *Methods Enzymol.* (1983) 91:324-332), or mild acid (Fontana et al., *Methods Enzymol.* (1983) 91:311-317), as described in the respective references.

5       Fragments generated from these digestion steps of *C. tinctorius* desaturase are presented in Fig. 1 and as SEQ ID NOS: 1-11.

10       Example 5

In this example, the preparation of a plant embryo cDNA bank, using the methods as described in Alexander, et al. (*Methods in Enzymology* (1987) 154:41-64) and the screening of the bank to obtain a desaturase cDNA clone is

15       described.

20       *C. tinctorius*: A plant embryo cDNA library may be constructed from poly(A)+ RNA isolated from *C. tinctorius* embryos collected at 14-17 days post-anthesis. Poly(A)+ RNA is isolated from polyribosomes by a method initially described by Jackson and Larkins (*Plant Physiol.* (1976) 57:5-10) as modified by Goldberg et al. (*Developmental Biol.* (1981) 83:201-217).

25       The plasmid cloning vector pCGN1703, derived from the commercial cloning vector Bluescribe M13- (Stratagene Cloning Systems; San Diego, CA), is made as follows. The polylinker of Bluescribe M13- is altered by digestion with *Bam*HI, treatment with mung bean endonuclease, and blunt-end ligation to create a *Bam*HI-deleted plasmid, pCGN1700. pCGN1700 is digested with *Eco*RI and *Sst*I (adjacent restriction sites) and annealed with synthetic complementary oligonucleotides having the sequences 5' CGGATCCACTGCAGTCTAGAGGGCCGGGA 3' (SEQ ID NO: 30) and 5' AATTTCCCGGGCCCTCTAGACTGCAGTGGATCCGAGCT 3' (SEQ ID NO: 31). These sequences are inserted to eliminate the *Eco*RI site, move the *Bam*HI site onto the opposite side of the *Sst*I (also, sometimes referred to as "SacI" herein) site found in Bluescribe, and to include new restriction sites *Pst*I, *Xba*I, *Apa*I, *Sma*I. The resulting plasmid pCGN1702, is

digested with *Hind*III and blunt-ended with Klenow enzyme; the linear DNA is partially digested with *Pvu*II and ligated with T4 DNA ligase in dilute solution. A transformant having the *lac* promoter region deleted is selected

5 (pCGN1703) and is used as the plasmid cloning vector.

Briefly, the cloning method for cDNA synthesis is as follows. The plasmid cloning vector is digested with *Sst*I and homopolymer T-tails are generated on the resulting 3'-overhang sticky-ends using terminal deoxynucleotidyl

10 transferase. The tailed plasmid is separated from undigested or un-tailed plasmid by oligo(dA)-cellulose chromatography. The resultant vector serves as the primer for synthesis of cDNA first strands covalently attached to either end of the vector plasmid. The cDNA-mRNA-vector

15 complexes are treated with terminal transferase in the presence of deoxyguanosine triphosphate, generating G-tails at the ends of the cDNA strands. The extra cDNA-mRNA complex, adjacent to the *Bam*HI site, is removed by *Bam*HI digestion, leaving a cDNA-mRNA-vector complex with a *Bam*HI

20 sticky-end at one end and a G-tail at the other. This complex is cyclized using the annealed synthetic cyclizing linker,

5'-

GATCCGGCGCGGAATTCGAGCTCCCCCCCCC-3' and

3'-GCGCCGGCGCTTAAGCTCGA-5'

25 which has a *Bam*HI sticky-end and a C-tail end. Following ligation and repair the circular complexes are transformed into *E. coli* strain DH5 $\alpha$  (BRL; Gaithersburg, MD) to generate the cDNA library. The *C. tinctorius* embryo cDNA bank contains between 3x10<sup>6</sup> and 5x10<sup>6</sup> clones with an average

30 cDNA insert size of approximately 1000 base pairs.

*Probe production Including PCR Reactions:* Two regions of amino acid sequence (Example 4) with low codon degeneracy are chosen from opposite ends of peptide sequence "Fragment F2" (SEQ ID NO:2) for production of a

35 probe for the plant desaturase cDNA. Two sets of mixed oligonucleotides are designed and synthesized for use as forward (SEQ ID NOS: 21-24) and reverse (SEQ ID NOS: 25-26) primers, respectively, in the polymerase chain reaction

(Saiki et al., *Science* (1985) 230:1350-1354; Oste, *Biotechniques* (1988) 6:162-167). See, Fig. 6. All oligonucleotides are synthesized on an Applied Biosystems 380A DNA synthesizer.

5 Probes to *C. tinctorius* desaturase may be prepared using the peptide sequence "Fragment 2" (SEQ ID NO: 2) identified in Fig. 1. Four types of forward primers were synthesized and labeled 13-1, 13-2, 13-3, and 13-4 (SEQ ID NOS: 21-24, respectively). Two groups of reverse primers 10 were synthesized and designated 13-5A and 13-6A (SEQ ID NOS: 25-26, respectively). The primer sequences are shown in Fig. 6. These oligonucleotide groups have a redundancy of 64 or less and contain either 20 or 17 bases of coding sequence along with flanking restriction site sequences for 15 *HindIII* or *EcoRI*. Based on the intervening amino acid sequence between the primer regions on peptide "Fragment 2" (SEQ ID NO: 2) the PCR product is expected to contain 107 base pairs.

20 Polymerase chain reaction is performed using the cDNA library DNA as template and the possible eight combinations of the four forward and two reverse oligonucleotides as primers in a Perkin-Elmer/Cetus DNA Thermal Cycler (Norwalk, CT) thermocycle file 1 min. 94°C, 2 min. 42°C, 2 min rise from 42°-72°C for 30 cycles, followed by the step 25 cycle file without step rises, 1 min. 94°C, 2 min. 42°C, 3 min. 72°C with increasing 15 sec extensions of the 72°C step for 10 cycles, and a final 10 min. 72°C extension.

30 The product of the 13-4 forward primer (SEQ ID NO: 24) and the 13-5A reverse primer (SEQ ID NO: 25) reaction was ethanol precipitated and then digested with *HindIII* and *EcoRI*, the resulting fragment was subcloned into pUC8 (Vieira and Messing, *Gene* (1982) 19:259-268).

35 Minipreparation DNA (Maniatis et al., *Molecular Cloning: A Laboratory Manual* (1982) Cold Harbor Laboratory, New York) of one clone was sequenced by Sanger dideoxy sequencing (Sanger et al., *Proc. Nat. Acad. Sci. USA* (1977) 74:5463-5467) using the M13 universal and reverse primers.

Translation of the resulting DNA sequence results in a

peptide sequence that exactly matches the amino acid sequence in peptide "Fragment F2" (SEQ ID NO: 2).

An exact 50 base oligonucleotide designated DESAT-50 is synthesized to match the sequence of the PCR reaction 5 product from the first valine residue to the last tyrosine residue.

The probe DSAT-50 5' -

GTAAGTAGGTAGGGCTTCCTCTGTAATCATATCTCCAACCAAAACAACAA -3' (SEQ 10 ID NO: 32) is used to probe the *C. tinctorius* embryo cDNA library.

#### *Library screen*

The *C. tinctorius* embryo cDNA bank is moved into the cloning vector lambda gt10 (Stratagene Cloning Systems) by 15 digestion of total cDNA with EcoRI and ligation to lambda gt10 DNA digested with EcoRI. The titer of the resulting library was  $\sim 5 \times 10^5$ /ml. The library is then plated on *E. coli* strain C600 (Huynh, et al., *DNA Cloning* Vol. 1 Eds. Glover D.M. IRL Press Limited: Oxford England, pp. 56, 110) 20 at a density of 5000 plaques/150 mm NZY ("NZYM" as defined in Maniatis et al. *supra*) agar plate to provide over 45,000 plaques for screening. Duplicate lifts are taken of the plaques using NEN Colony Plaque Screen filters by laying precut filters over the plates for  $\sim 1$  minute and then 25 peeling them off. The phage DNA is immobilized by floating the filters on denaturing solution (1.5M NaCl, .05M NaOH) for 1 min., transferring the filters to neutralizing solution (1.5M NaCl, 0.5M Tris-HCl pH 8.0) for 2 min. and then to 2XSSC (1xSSC = 0.15M NaCl; 0.015M Na citrate) for 30 min., followed by air drying. The filters are hybridized with  $^{32}P$  end-labeled DSAT-50 oligonucleotide (SEQ ID NO: 32) (BRL 5' DNA Terminus Labeling System) by the method of Devlin et al., (*DNA* (1988) 7:499-807) at 42° C overnight, and washed for 30 min. at 50°C in 2XSSC, 0.5% SDS and then 35 twice for 20 min. each at 50°C in 0.1XSSC, 0.5% SDS. Filters are exposed to X-ray film at -70°C with a Dupont Cronex intensifying screen for 48 hours.

Clones are detected by hybridization with the DSAT-50 oligonucleotide and plaque purified. The complete nucleotide sequence (SEQ ID NO: 12) of the cDNA insert of a clone, pCGN2754, and a partial restriction map thereof are 5 presented in Figures 2 and 7A, respectively. The cDNA insert includes 1533 bases plus a poly(A) track at the 3' end of 100-200 bases. The open reading frame for the desaturase begins at the first ATG (nucleotide 106) from the 5' end and stops at nucleotide 1294. The translated 10 amino acid sequence is presented in Fig. 2 and SEQ ID NO: 13. The open reading frame includes a 33 amino acid transit peptide not found in the amino acid sequence of the mature protein. The N-terminus of the protein begins at the alanine immediately following the NcoI site (nucleotide 15 202) indicating the site of the transit peptide processing.

Example 6

In this example, expression of a plant desaturase in a prokaryote is described.

20 *Desaturase expression construct in E. coli*

A plasmid for expression of desaturase activity in *E. coli* is constructed as follows. The desaturase cDNA clone pCGN2754 is digested with *Hind*III and *Sal*I and ligated to pCGN2016 (a chloramphenicol resistant version of Bluescript 25 KS-) digested with *Hind*III and *Xho*I. The resulting plasmid is pCGN1894.

pCGN2016 is prepared by digesting pCGN565 with *Hha*I, and the fragment containing the chloramphenicol resistance gene is excised, blunted by use of mung bean nuclease, and 30 inserted into the *Eco*RV site of Bluescript KS- (Stratagene: La Jolla, CA) to create pCGN2008. The choramphenicol resistance gene of pCGN2008 is removed by *Eco*RI/*Hind*III digestion. After treatment with Klenow enzyme to blunt the ends, the fragment is ligated to *Dra*I digested Bluescript 35 KS-. A clone that has the *Dra*I fragment containing ampicillin resistance replaced with the chloramphenicol resistance is chosen and named pCGN2016.

pCGN565 is a cloning vector based on pUC12-cm (K. Buckley Ph.D. Thesis, Regulation and expression of the phi X174 lysis gene, University of California, San Diego, 1985), but contains pUC18 linkers (Yanisch-Perron, et al., Gene (1985) 53:103-119).

5 The fragment containing the mature coding region of the  $\Delta$ -9 desaturase, 3'-noncoding sequences and poly(A) tails is subcloned from pCGN1894 digested with NcoI and Asp718 into pUC120, an *E. coli* expression vector based on pUC118 (Vieira and Messing, *Methods in Enzymology* (1987) 10 153:3-11) with the lac region inserted in the opposite orientation and an NcoI site at the ATG of the lac peptide (Vieira, J. PhD. Thesis, University of Minnesota, 1988). The *E. coli* desaturase expression plasmid is designated 15 pCGN3201. The desaturase sequences are inserted such that they are aligned with the lac transcription and translation signals.

#### *Expression of Desaturase in E.coli*

20 Single colonies of *E. coli* strain 7118 (Maniatis et al., *supra*) containing pUC120 or pCGN3201 are cultured in 80 mls each of ECLB broth, 300 mg/L penicillin. The cells are induced by the addition of 1mM IPTG. Cells are grown overnight (18 hrs) at 37° C.

25 Eighty mls of overnight cultures of *E. coli* (induced and uninduced) containing pUC120 or pCGN3201 are centrifuged at 14,800 x g for 15 min. The pelleted cells are resuspended in 3 mls 20 mM phosphate buffer, pH 6.8. Resuspended cells were broken in a french press at 16,000 psi. Broken cell mixtures are centrifuged 5000xg for 5 30 min. 100  $\mu$ l of each supernatant is applied to a G-25 Sephadex gel filtration centrifugal column (Boehringer Mannheim Biochemicals), equilibrated in 20mM phosphate buffer pH 6.8. Columns are spun for 4 min at 5000xg. Effluent was collected and used as enzyme source in the 35 desaturase assay.

Desaturase activity is assayed as described in Example 1. Both pUC120-containing, IPTG-induced cells and uninduced cells do not express detectable stearoyl-ACP

desaturase activity. The pCGN3201 IPTG-induced extract contains 8.22 nmol/min of desaturase activity. pCGN3201 uninduced extracts contains 6.45 nmol/min of activity. The pCGN3201 IPTG-induced extract shows 21.5% more activity  
5 than the uninduced pCGN3201 extract.

*Detection of induced protein in E. coli.*

Extracts of overnight cultures of *E. coli* strain 7118 (Maniatis et al. *supra*) containing pCGN3201 or pUC120  
10 grown in ECLB containing 300 mg/L penicillin induced with 1mM IPTG are prepared as follows. 1.5 ml of overnight culture grown shaking at 37°C are pelleted in Eppendorf tubes for 10 min at 10-13,000 µg. Pellets are resuspended in 150 ul SDS sample buffer (0.05M Tris-HCl, pH6.8, 1% SDS,  
15 5% β-mercaptoethanol, 10% glycerol and 0.005% bromophenol blue) and boiled for 10 min. 25 µl of each sample are electrophoresed on a 10% polyacrylamide gel (Laemmli, *Nature* (1970) 227:680) at 25 mA for 5 hours. Gels are stained in 0.05% Coomassie Brilliant Blue, 25% isopropanol  
20 and 10% acetic acid and destained in 10% acetic acid and 10% isopropanol. A band is detected at a position just below the 43,000 MW protein marker (SDS PAGE standard, Low molecular weight, BioRad, Richmond CA) in the pCGN3201 extracts that is not present in the pUC120 extracts. This  
25 is the approximate molecular weight of mature desaturase protein.

*Requirement for Spinach Ferredoxin*

Stearoyl-ACP desaturase can also be expressed in *E.*  
30 *coli* by subcloning into the *E. coli* expression vector pET8c (Studier, et al., *Methods Enzymol.* (1990) 185:60-89). The mature coding region (plus an extra Met codon) of the desaturase cDNA with accompanying 3'-sequences is inserted as an *Ncol* - *Sma* 1 fragment into pET8c at the *Ncol* and  
35 *Bam*H1 sites (after treatment of the *Bam*H1 site with Klenow fragment of DNA polymerase to create a blunt end) to create pCGN3208. The plasmid pCGN3208 is maintained in *E. coli* strain BL21(DE3) which contains the T7 polymerase gene

under the control of the isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG)-inducible lacUV5 promoter (Studier et al., *supra*).

5 *E. coli* cells containing pCGN3208 are grown at 37°C to an OD<sub>595</sub> of ~0.7 in NZY broth containing 0.4% (w/v) glucose and 300 mg/liter penicillin, and then induced for 3 hours with 0.4 mM IPTG. Cells are pelleted from 1 ml of culture, dissolved in 125  $\mu$ l of SDS sample buffer (10) and heated to 100°C for 10 min. Samples are analyzed by SDS

10 polyacrylamide gel electrophoresis at 25 mA for 5 h. Gels are stained in 0.05% Coomassie Brilliant Blue, 25% (v/v) isopropanol and 10% (v/v) acetic acid. A band is detected at a position just below the 43,000 MW protein marker (SDS PAGE standard, Low Molecular Weight, BioRad, Richmond, CA)

15 in the pCGN3208 extract that is not present in the pET8c extracts. This is the approximate molecular weight of mature desaturase protein.

For activity assays, cells are treated as described above and extracts are used as enzyme source in the

20 stearoyl-ACP desaturase assay as described in Example 1. The extract from IPTG-induced pCGN3208 cells contains 8.61 nmol/min/mg protein of desaturase activity. The extract from pCGN3208 uninduced cells contains 1.41 nmol/min/mg protein of desaturase activity. The extract from pCGN3208

25 induced cells, thus has approximately 6-fold greater activity than the extract from uninduced pCGN3208 cells. Extracts from both induced and uninduced cells of pET8c do not contain detectable stearoyl-ACP desaturase activity.

Samples are also assayed as described in Example 1,

30 but without the addition of spinach ferredoxin, to determine if the *E. coli* ferredoxin is an efficient electron donor for the desaturase reaction. Minimal activity is detected in *E. coli* extracts unless spinach ferredoxin is added exogenously.

Example 7

In this example, the preparation of an ACP expression cassette containing a plant desaturase in a binary vector suitable for plant transformation is described.

5

**ACP Expression Cassette**

An expression cassette utilizing 5'-upstream sequences and 3'-downstream sequences obtainable from *B. campestris* ACP gene can be constructed as follows.

10 A 1.45kb *Xho*I fragment of Bcg 4-4 (Fig. 9 and SEQ ID NO: 28) containing 5'-upstream sequences is subcloned into the cloning/sequencing vector Bluescript+ (Stratagene Cloning Systems, San Diego, CA). The resulting construct, pCGN1941, is digested with *Xho*I and ligated to a  
15 chloramphenicol resistant Bluescript M13+ vector, pCGN2015 digested with *Xho*I. pCGN2015 is prepared as described for pCGN2016 (See, Example 6) except that the *Eco*RI/*Hind*III "chloramphenicol" fragment isolated from pCGN2008 is ligated with the 2273 bp fragment of Bluescript KS<sup>+</sup>  
20 (Stratagene; LaJolla, CA) isolated after digestion with *Dra*I. This alters the antibiotic resistance of the plasmid from penicillin resistance to chloramphenicol resistance. The chloramphenicol resistant plasmid is pCGN1953.

25 3'-sequences of Bcg 4-4 are contained on an *Sst*I/*Bgl*II fragment cloned in the *Sst*I/*Bam*HI sites of M13 Bluescript+ vector. This plasmid is named pCGN1940. pCGN1940 is modified by *in vitro* site-directed mutagenesis (Adelman et al., *DNA* (1983) 2:183-193) using the synthetic oligonucleotide 5'-CTTAAGAAGTAACCCGGGCTGCAGTTTAGTATTAAGAG-  
30 3' (SEQ ID NO: 33) to insert *Sma*I and *Pst*I restriction sites immediately following the stop codon of the reading frame for the ACP gene 18 nucleotides from the *Sst*I site. The 3'-noncoding sequences from this modified plasmid, pCGN1950, are moved as a *Pst*I-*Sma*I fragment into pCGN1953  
35 cut with *Pst*I and *Sma*I. The resulting plasmid pCGN1977 comprises the ACP expression cassette with the unique restriction sites *Eco*RV, *Eco*RI and *Pst*I available between the 1.45kb 5' and 1.5 kb of 3'-noncoding sequences (SEQ ID

NO: 28) for the cloning of genes to be expressed under regulation of these ACP gene regions.

#### *Desaturase Expression in Plants*

5       Desaturase cDNA sequences from pCGN2754 are inserted in the ACP expression cassette, pCGN1977, as follows. pCGN2754 is digested with *Hind*III (located 160 nucleotides upstream of the start codon) and *Asp*718 located in the polylinker outside the poly(A) tails. The fragment  
10      containing the coding region for desaturase was blunt-ended using DNA polymerase I and ligated to pCGN1977 digested with *Eco*RV. A clone containing the desaturase sequences in the sense orientation with respect to the ACP promoter is selected and called pCGN1895. This expression cassette may  
15      be inserted into a binary vector, for example, for *Agrobacterium*-mediated transformation, or employed in other plant transformation techniques.

#### *Binary Vector and Agrobacterium Transformation*

20       The fragment containing the pCGN1895 expression sequences ACP 5'/desaturase/ACP 3' is cloned into a binary vector pCGN1557 (described below) for *Agrobacterium* transformation by digestion with *Asp*718 and *Xba*I and ligation to pCGN1557 digested with *Asp*718 and *Xba*I. The  
25      resulting binary vector is called pCGN1898.

pCGN1898 is transformed into *Agrobacterium tumefaciens* strain EHA101 (Hood, et al., *J. Bacteriol.* (1986) 168:1291-1301) as per the method of Holsters, et al., *Mol. Gen. Genet.* (1978) 163:181-187.

30       RNA blot analysis of seeds (T2) from T1 plants show the presence of a mRNA species for the inserted *C. tinctorius* desaturase, but the amount of message is low compared to endogenous levels of mRNA for the *Brassica* desaturase, suggesting that the expression levels were  
35      insufficient to significantly increase the amount of desaturase enzyme above that normally present. This is consistent with the negative results from oil, desaturase activity and Western blot analyses.

Construction of pCGN1557

pCGN1557 (McBride and Summerfelt, *Plant Molecular Biology* (1990) 14(2):269-276) is a binary plant transformation vector containing the left and right T-DNA borders of *Agrobacterium tumefaciens* octopine Ti-plasmid pTiA6 (Currier and Nester, *supra*, the gentamycin resistance gene of pPH1JI (Hirsch and Beringer, *supra*), an *Agrobacterium rhizogenes* Ri plasmid origin of replication from pLJbB11 (Jouanin et al., *supra*), a 35S promoter-kanR-tml3' region capable of conferring kanamycin resistance to transformed plants, a ColE1 origin of replication from pBR322 (Bolivar et al., *supra*), and a lacZ' screenable marker gene from pUC18 (Yanish-Perron et al., *supra*).

There are three major intermediate constructs used to generate pCGN1557:

pCGN1532 (see below) contains the pCGN1557 backbone, the pRI plasmid origin of replication, and the ColE1 origin of replication.

pCGN1546 (see below) contains the CaMV35S5'-kanR-tml3' plant selectable marker region.

pCGN1541b (see below) contains the right and left T-DNA borders of the *A. tumefaciens* octopine Ti-plasmid and the lacZ' region from pUC19.

To construct pCGN1557 from the above plasmids, pCGN1546 is digested with *Xba*I, and the fragment containing the CaMV 35S5'-kanR-tml3' region is cloned into the *Xba*I site of pCGN1541b to give the plasmid pCGN1553, which contains T-DNA/left border/CaMV 35S5'-kanR-tml3'/lacZ'/T-DNA left border. pCGN1553 is digested with *Bgl*II, and the fragment containing the T-DNA/left border/CaMV35S5'-kanR-tml3'/lacZ'/T-DNA left border region is ligated into *Bam*HI-digested pCGN1532 to give the complete binary vector, pCGN1557.

35        pCGN1532

The 3.5kb *Eco*RI-*Pst*I fragment containing the gentamycin resistance gene is removed from pPH1JI (Hirsch and Beringer, *Plasmid* (1984) 12:139-141) by *Eco*RI-*Pst*I

digestion and cloned into *EcoRI-PstI* digested pUC9 (Vieira and Messing, *Gene* (1982) 19:259-268) to generate pCGN549. *HindIII-PstI* digestion of pCGN549 yields a 3.1 kb fragment bearing the gentamycin resistance gene, which is made blunt 5 ended by the Klenow fragment of DNA polymerase I and cloned into *PvuII* digested pBR322 (Bolivar et al., *Gene* (1977) 2:95-113) to create pBR322Gm. pBR322Gm is digested with *DraI* and *SphI*, treated with Klenow enzyme to create blunt ends, and the 2.8 kb fragment cloned into the *Ri* origin-10 containing plasmid pLJbB11 (Jouanin et al., *Mol. Gen. Genet.* (1985) 201:370-374) which has been digested with *ApaI* and made blunt-ended with Klenow enzyme, creating pLHbB11Gm. The extra *ColE1* origin and the kanamycin resistance gene are deleted from pLHbB11Gm by digestion 15 with *BamHI* followed by self closure to create pGmB11. The *HindII* site of pGmB11 is deleted by *HindIII* digestion followed by treatment with Klenow enzyme and self closure, creating pGmB11-H. The *PstI* site of pGmB11-H is deleted by *PstI* digestion followed by treatment with Klenow enzyme and 20 self closure, creating pCGN1532.

#### Construction of pCGN1546

The 35S promoter-tml3' expression cassette, pCGN986, contains a cauliflower mosaic virus 35S (CaMV35) promoter 25 and a T-DNA tml 3'-region with multiple restriction sites between them. pCGN986 is derived from another cassette, pCGN206, containing a CaMV35S promoter and a different 3' region, the CaMV region VI 3'-end. The CaMV 35S promoter is cloned as an *AluI* fragment (bp 7144-7734) (Gardner et. 30 al., *Nucl. Acids Res.* (1981) 9:2871-2888) into the *HincII* site of M13mp7 (Messing, et. al., *Nucl. Acids Res.* (1981) 9:309-321) to create C614. An *EcoRI* digest of C614 produced the *EcoRI* fragment from C614 containing the 35S 35 promoter which is cloned into the *EcoRI* site of pUC8 (Vieira and Messing, *Gene* (1982) 19:259) to produce pCGN147.

pCGN148a containing a promoter region, selectable marker (KAN with 2 ATG's) and 3' region, is prepared by

digesting pCGN528 with *Bgl*II and inserting the *Bam*HI-*Bgl*II promoter fragment from pCGN147. This fragment is cloned into the *Bgl*II site of pCGN528 so that the *Bgl*II site is proximal to the kanamycin gene of pCGN528.

5 The shuttle vector used for this construct pCGN528, is made as follows: pCGN525 is made by digesting a plasmid containing Tn5 which harbors a kanamycin gene (Jorgenson et. al., *Mol. Gen. Genet.* (1979) 177:65) with *Hind*III-*Bam*HI and inserting the *Hind*III-*Bam*HI fragment containing the 10 kanamycin gene into the *Hind*III-*Bam*HI sites in the tetracycline gene of pACYC184 (Chang and Cohen, *J. Bacteriol.* (1978) 134:1141-1156). pCGN526 was made by inserting the *Bam*HI fragment 19 of pTiA6 (Thomashow et. al., *Cell* (1980) 19:729-739), modified with *Xba*I linkers 15 inserted into the *Sma*I site, into the *Bam*HI site of pCGN525. pCGN528 is obtained by deleting the small *Xba*I fragment from pCGN526 by digesting with *Xba*I and religating.

20 pCGN149a is made by cloning the *Bam*HI-kanamycin gene pMB9KanXXI into the *Bam*HI site of pCGN148a. pMB9KanXXI is a pUC4K variant (Vieira and Messing, *Gene* (1982) 19:259-268) which has the *Xba*I site missing, but contains a functional kanamycin gene from Tn903 to allow for efficient selection in *Agrobacterium*.

25 pCGN149a is digested with *Hind*III and *Bam*HI and ligated to pUC8 digested with *Hind*III and *Bam*HI to produce pCGN169. This removes the Tn903 kanamycin marker. pCGN565 (see pCGN2016 description) and pCGN169 are both digested with *Hind*III and *Pst*I and ligated to form pCGN203, a 30 plasmid containing the CaMV 35S promoter and part of the 5'-end of the Tn5 kanamycin gene (up to the *Pst*I site, Jorgenson et. al., (1979), *supra*). A 3'-regulatory region is added to pCGN203 from pCGN204 (an *Eco*RI fragment of CaMV (bp 408-6105) containing the region VI 3' cloned into pUC18 35 (Yanisch-Perron, et al., *Gene* (1985) 33:103-119) by digestion with *Hind*III and *Pst*I and ligation. The resulting cassette, pCGN206, is the basis for the construction of pCGN986.

The pTiA6 T-DNA *tml* 3'-sequences are subcloned from the *Bam*19 T-DNA fragment (Thomashow et al., (1980) *supra*) as a *Bam*HI-*Eco*RI fragment (nucleotides 9062 to 12,823, numbering as in Barker et al., *Plant Mol. Biol.* (1982)

5 2:335-350) and combined with the pACYC184 (Chang and Cohen (1978), *supra*) origin of replication as an *Eco*RI-*Hind*III fragment and a gentamycin resistance marker (from plasmid pLB41), obtained from D. Figurski) as a *Bam*HI-*Hind*III fragment to produce pCGN417.

10 The unique *Sma*I site of pCGN417 (nucleotide 11,207 of the *Bam*19 fragment) is changed to a *Sac*I site using linkers and the *Bam*HI-*Sac*I fragment is subcloned into pCGN565 to give pCGN971. The *Bam*HI site of pCGN971 is changed to an *Eco*RI site using linkers. The resulting *Eco*RI-*Sac*I 15 fragment containing the *tml* 3' regulatory sequences is joined to pCGN206 by digestion with *Eco*RI and *Sac*I to give pCGN975. The small part of the *Tn*5 kanamycin resistance gene is deleted from the 3'-end of the CaMV 35S promoter by digestion with *Sal*I and *Bgl*III, blunting the ends and 20 ligation with *Sal*I linkers. The final expression cassette pCGN986 contains the CaMV 35S promoter followed by two *Sal*I sites, an *Xba*I site, *Bam*HI, *Sma*I, *Kpn*I and the *tml* 3' region (nucleotides 11207-9023 of the T-DNA).

25 The 35S promoter-*tml* 3' expression cassette, pCGN986 is digested with *Hind*III. The ends are filled in with Klenow polymerase and *Xho*I linkers added. The resulting plasmid is called pCGN986X. The *Bam*HI-*Sac*I fragment of pBRX25 (see below) containing the nitrilase gene is inserted into *Bam*HI-*Sac*I digested pCGN986X yielding pBRX66.

30 Construction of pBRX25 is described in U.S. Letters Patent 4,810,648, which is hereby incorporated by reference. Briefly, the method is as follows: The nucleotide sequence of a 1212-bp *Pst*I-*Hinc*II DNA segment encoding the bromoxynil-specific nitrilase contains 65-bp 35 of 5' untranslated nucleotides. To facilitate removal of a portion of these excess nucleotides, plasmid pBRX9 is digested with *Pst*I, and treated with nuclease *Bal*31. *Bam*HI linkers are added to the resulting ends. *Bam*HI-*Hinc*II

fragments containing a functional bromoxynil gene are cloned into the *Bam*HI-*Sma*I sites of pCGN565. The resulting plasmid, pBRX25, contains only 11 bp of 5' untranslated bacterial sequence.

5 pBRX66 is digested with *Pst*I and *Eco*RI, blunt ends generated by treatment with Klenow polymerase, and *Xho*I linkers added. The resulting plasmid pBRX68 now has a tml 3' region that is approximately 1.1kb. pBRX68 is digested with *Sal*I and *Sac*I, blunt ends generated by treatment with 10 Klenow polymerase and *Eco*RI linkers added. The resulting plasmid, pCGN986XE is a 35S promoter - tml 3' expression cassette lacking the nitrilase gene.

The Tn5 kanamycin resistance gene is then inserted into pCGN986XE. The 1.0 kb *Eco*RI fragment of pCGN1536 (see 15 pCGN1547 description) is ligated into pCGN986XE digested with *Eco*RI. A clone with the Tn5 kanamycin resistance gene in the correct orientation for transcription and translation is chosen and called pCGN1537b. The 35S promoter Kan<sup>R</sup>-tml 3' region is then transferred to a 20 chloramphenical resistant plasmid backbone. pCGN786, (a pUC-CAM based vector with the synthetic oligonucleotide 5' GGAATTCGTCGACAGATCTCTGCAGCTCGAGGGATCCAAGCTT 3' (SEQ ID NO: 34) containing the cloning sites *Eco*RI, *Sal*I, *Bgl*III, *Pst*I, *Xho*I, *Bam*HI, and *Hind*III inserted into pCGN566, pCGN566 25 contains the *Eco*HI-*Hind*III linker of pUC18 inserted into the *Eco*KI-*Hind*III sites of pUC13-cm (K. Buckler (1985) *supra*) is digested with *Xho*I and the *Xho*I fragment of pCGN1537b containing the 35S promoter - Kan<sup>R</sup>-tml 3' region is ligated in. The resulting clone is termed pCGN1546.

30

**pCGN1541b**

pCGN565RB02X (see below) is digested with *Bgl*III and *Xho*I, and the 728bp fragment containing the T-DNA right border piece and the *lacZ'* gene is ligated with *Bgl*III-*Xho*I 35 digested pCGN65ΔKX-S+K (see below), replacing the *Bgl*III-*Xho*I right border fragment of pCGN65ΔKX-S+K. The resulting plasmid, pCGN65α2X contains both T-DNA borders and the *lacZ'* gene. The *Cla*I fragment of pCGN65α2X is

replaced with an *Xba*I site by digesting with *Cla*I blunting the ends using the Klenow fragment, and ligating with *Xba*I linker DNA, resulting in plasmid pCGN65α2XX. pCGN65α2XX is digested with *Bgl*III and *Eco*RV, treated with the Klenow 5 fragment of DNA polymerase I to create blunt ends, and ligated in the presence of *Bgl*III linker DNA, resulting in pCGN65α2XX'. pCGN65α2XX' is digested with *Bgl*III and ligated with *Bgl*III digested pCGN1538 (see below), resulting in pCGN1541a, which contains both plasmid backbones.

10 pCGN1541a is digested with *Xba*I and religated. Ampicillin resistant, chloramphenicol sensitive clones are chosen, which lack the pACYC184-derived backbone, creating pCGN1541b.

pCGN1538 is generated by digesting pBR322 with *Eco*RI 15 and *Pvu*II, treating with Klenow to generate blunt ends, and ligating with *Bgl*III linkers. pCGN1538 is ampicillin resistant, tetracycline sensitive.

**pCGN65ΔXX-S+K**

20 pCGN501 is constructed by cloning a 1.85 kb *Eco*RI-*Xba*I fragment of pTiA6 (Currier and Nester, *J. Bact.* (1976) 126:157-165) containing bases 13362-15208 (Barker et al., *Plant Mo. Biol.* (1983) 2:335-350) of the T-DNA (right border), into *Eco*RI-*Sal*I digested M13mp9 (Vieira and 25 Messing, *Gene* (1982) 19:259-268). pCGN502 is constructed by cloning a 1.6 kb *Hind*III-*Sma*I fragment of pTiA6, containing bases 602-2212 of the T-DNA (left border), into *Hind*III-*Sma*I digested M13mp9. pCGN501 and pCGN502 are both digested with *Eco*RI and *Hind*III and both T-DNA-containing 30 fragments cloned together into *Hind*III digested pUC9 (Vieira and Messing, *Gene* (1982) 19:259-268) to yield pCGN503, containing both T-DNA border fragments. pCGN503 is digested with *Hind*III and *Eco*RI and the two resulting *Hind*III-*Eco*RI fragments (containing the T-DNA borders) are 35 cloned into *Eco*RI digested pHC79 (Hohn and Collins, *Gene* (1980) 11:291-298) to generate pCGN518. The 1.6kb *Kpn*I-*Eco*RI fragment from pCGN518, containing the left T-DNA border, is cloned into *Kpn*I-*Eco*RI digested pCGN565 to

generate pCGN580. The *Bam*III-*Bgl*III fragment of pCGN580 is cloned into the *Bam*HI site of pACYC184 (Chang and Cohen, *J. Bacteriol.* (1978) 134:1141-1156) to create pCGN51. The 1.4 kb *Bam*HI-*Sph*I fragment of pCGN60 containing the T-DNA right border fragment, is cloned into *Bam*HI-*Sph*I digested pCGN51 to create pCGN65, which contains the right and left T-DNA borders.

5 pCGN65 is digested with *Kpn*I and *Xba*I, treated with Klenow enzyme to create blunt ends, and ligated in the presence of synthetic *Bgl*III linker DNA to create pCGN65 $\Delta$ KX. pCGN65 $\Delta$ KX is digested with *Sal*I, treated with Klenow enzyme to create blunt ends, and ligated in the presence of synthetic *Xho*I linker DNA to create pCGN65 $\Delta$ KX-S+X.

15 pCGN565RB $\alpha$ 2X

pCGN451 (see below) is digested with *Hpa*I and ligated in the presence of synthetic *Sph*I linker DNA to generate pCGN55. The *Xho*I-*Sph*I fragment of pCGN55 (bp13800-15208, including the right border, of *Agrobacterium tumefaciens* T-DNA; (Barker et al., *Gene* (1977) 2:95-113) is cloned into *Sal*I-*Sph*I digested pUC19 (Yanisch-Perron et al., *Gene* (1985) 53:103-119) to create pCGN60. The 1.4 kb *Hind*III-*Bam*HI fragment of pCGN60 is cloned into *Hind*III-*Bam*HI digested pSP64 (Promega, Inc.) to generate pCGN1039. pCGN1039 is digested with *Sma*I and *Nru*I (deleting bp14273-15208; (Barker et al., *Gene* (1977) 2:95-113) and ligated in the presence of synthetic *Bgl*III linker DNA creating pCGN1039 $\Delta$ NS. The 0.47 kb *Eco*RI-*Hind*III fragment of pCGN1039 $\Delta$ NS is cloned into *Eco*RI-*Hind*III digested pCGN565 to create pCGN565RB. The *Hind*III site of pCGN565RB is replaced with an *Xho*I site by digesting with *Hind*III, treating with Klenow enzyme, and ligating in the presence of synthetic *Xho*I linker DNA to create pCGN565RB-H+X.

30 35 pUC18 (Norrrander et al., *Gene* (1983) 26:101-106) is digested with *Hae*II to release the *lacZ*' fragment, treated with Klenow enzyme to create blunt ends, and the *lacZ*'-containing fragment ligated into pCGN565RB-H+X, which had been digested with *Acc*I and *Sph*I and treated with Klenow

enzyme in such a orientation that the *lacZ'* promoter is proximal to the right border fragment; this construct, pCGN565RB $\alpha$ 2x is positive for *lacZ'* expression when plated on an appropriate host and contains bp 13990-14273 of the 5 right border fragment (Barker et al., *Plant Mo. Biol.* (1983) 2:335-350) having deleted the *AccI-SphI* fragment (bp 13800-13990).

pCGN451

pCGN451 contains an *ocs5'-ocs3'* cassette, including 10 the T-DNA right border, cloned into a derivative of pUC8 (Vieira and Messing, *supra*). The modified vector is derived by digesting pUC8 with *HincII* and ligating in the presence of synthetic linker DNA, creating pCGN416, and then deleting the *EcoRI* site of pCGN416 by *EcoRI* digestion 15 followed by treatment with Klenow enzyme and self-ligation to create pCGN426.

The *ocs5'-ocs3'* cassette is created by a series of steps from DNA derived from the octopine Ti-plasmid pTiA6 (Currier and Nester, *supra*). To generate the 5'end, which 20 includes the T-DNA right border, an *EcoRI* fragment of pTiA6 (bp 13362-16202 (the numbering is by Barker, et al., (*Plant Mol. Bio* (1983) 2:335-350) for the closely related Ti plasmid pTi15955)) is removed from pVK232 (Knauf and Nester, *Plasmid* (1982) 8:45) by *EcoRI* digestion and cloned 25 into *EcoRI* digested pACYC184 (Chang and Cohen, *supra*) to generate pCGN15.

The 2.4kb *BamHI-EcoRI* fragment (bp 13774-16202) of pCGN15 is cloned into *EcoRI-BamHI* digested pBR322 (Bolivar, et al., *supra*) to yield pCGN429. The 412 bp *EcoRI-BamHI* 30 fragment (bp 13362-13772) of pCGN15 is cloned into *EcoRI-BamHI* digested pBR322 to yield pCGN407. The cut-down promoter fragment is obtained by digesting pCGN407 with *XmnI* (bp 13512), followed by resection with *Bal31* exonuclease, ligation of synthetic *EcoRI* linkers, and 35 digestion with *BamHI*. Resulting fragments of approximately 130 bp are gel purified and cloned into M13mp9 (Vieira and Messing, *supra*) and sequenced. A clone, I-4, in which the *EcoRI* linker has been inserted at bp 1362 between the

transcription initiation point and the translation initiation codon is identified by comparison with the sequence of de Greve, et al., (J. Mol. Appl. Genet. (1982) 1:499-512). The EcoRI cleavage site is at position 13639, 5 downstream from the mRNA start 'site. The 141 bp EcoRI-BamHI fragment of I-4, containing the cut-down promoter, is cloned into EcoRI-BamHI digested pBR322 to create pCGN428. The 141 bp EcoRI-BamHI promoter piece from pCGN428, and the 2.5 kb EcoRI-BamHI ocs5' piece from pCGN429 are cloned 10 together into EcoRI digested pUC19 (Vieira and Messing, *supra*) to generate pCGN442, reconstructing the ocs upstream region with a cut-down promoter section.

To generate the ocs3' end, the HindIII fragment of pLB41 (D. Figurski, UC San Diego) containing the gentamycin 15 resistance gene is cloned into HindIII digested pACYC184 (Chang and Cohen, *supra*) to create pCGN413b. The 4.7 kb BamHI fragment of pTiA6 (*supra*), containing the ocs3' region, is cloned into BamHI digested pBR325 (F. Bolivar, Gene (1978) 4:121-136) to create 33c-19. The SmaI site at 20 position 11207 (Barker, *supra*) of 33c-19 is converted to an XhoI site using a synthetic XhoI linker, generating pCCG401.2. The 3.8 kb BamHI-EcoRI fragment of pCGN401.2 is cloned into BamHI-EcoRI digested pCGN413b to create pCGN419.

25 The ocs5'-ocs3' cassette is generated by cloning the 2.64 kb EcoRI fragment of pCGN442, containing the 5' region, into EcoRI digested pCGN419 to create pCNG446. The 3.1kb XhoI fragment of pCNG446, having the ocs5' region (bp 13639-15208) and ocs3' region (bp 11207-12823), is cloned 30 into the XhoI site of pCGN426 to create pCGN451.

#### Example 8

In this example, the preparation of a Bce-4 expression cassette containing a plant desaturase is described.

35 The desaturase cDNA clone from pCGN2754 prepared as described in Example 5, is modified by *in vitro* mutagenesis to insert restriction sites immediately upstream of the ATG start codon and downstream of the TGA stop codon. A

single-stranded template DNA is prepared for the mutagenesis reaction from pCGN1894 (described in Example 6) as described by Messing, (Methods in Enzymol. (1983) 101:20-79). Synthetic oligonucleotides are synthesized on 5 an Applied Biosystems 380A DNA synthesizer. The oligonucleotides used are

5'-CCATTTTGATCTCCTCGAGCCGGGCTGCAGTTCTTCTTCTTGTG-3'  
(SEQ ID NO: 35) for the 5' mutagenesis and  
5'-GCTCGTTTTTTCTCTGCAGCCGGGCTCGAGTCACAGCTTCACC -3'  
10 (SEQ ID NO: 36) for the 3'-mutagenesis; both add *Pst*I, *Sma*I and *Xho*I sites flanking the coding region. Both oligonucleotides are 5'-phosphorylated (BRL 5'-Terminus labelling kit) and used for mutagenesis with the pCGN1894 template by the procedure of Adelman et al. (DNA (1983) 2:183-193). Alternatively, the desired restriction sites 15 may be inserted by PCR, using the 3' oligo described above (SEQ ID NO: 36) and another oligo,  
5' ACTGACTGCAGCCGGGCTCGAGGAAGATCAAAATGGCTCTTC 3' (SEQ ID NO: 37) for the 3' and 5' primers, respectively. The 20 template in this polymerase chain reaction is DNA from pCGN1894. The *Xho*I fragment from the resulting clone can be subcloned into the *Bce*4 expression cassette, pCGN1870 (described below) at the unique *Xho*I site. This 25 *Bce*4/desaturase expression cassette can then be inserted in a suitable binary vector, transformed into *Agrobacterium tumefaciens* strain EHA101 and used to transform plants as provided in Example 10.

#### Bce-4 Expression Cassette

30 pCGN1870 is a *Bce*-4 expression cassette containing 5' and 3' regulatory regions of the *Bce*-4 gene and may be derived from the *Bce*-4 sequence found in pCGN1857, which was deposited with the ATCC on March 9, 1990, and assigned 35 accession number 68251, or by methods known to one skilled in the art from the sequence (SEQ ID NO: 27) provided in Fig. 8. The *Bce* 4 gene may be isolated as follows:

The *Cla*I fragment of pCGN1857, containing the *Bce*4 gene is ligated into *Cla*I digested Bluescript KS+

(Stratagene; La Jolla, CA), producing pCGN1864. Single stranded DNA is made from pCGN1864 and altered by *in vitro* mutagenesis using the oligonucleotides

BCE45P:

5 (5'GAGTAGTGAACCTTCATGGATCCTCGAGGTCTTGAAAACCTAGA3') (SEQ ID NO: 38) and

BCE43P:

(5'CAATGTCTTGAGAGATCCCGGGATCCTTAACAACTAGGAAAAGG3') (SEQ ID NO: 39)

10 as described by Adelman *et al.* (DNA (1983) 2:183-193). The oligonucleotide BSCP2 (5'GTAAGACACGACTTATGCCACTG3') (SEQ ID NO: 40), complementary to a portion of Bluescript, is included in the reaction to improve the yield of double-stranded DNA molecules. The resulting plasmid, pCGN1866, 15 contains *Xho*I and *Bam*HI sites (from BCE45P) immediately 5' to the Bce4 start codon and *Bam*HI and *Sma*I sites (from BCE43P) immediately 3' to the Bce4 stop codon. The *Cla*I fragment of pCGN1866, containing the mutagenized sequences, is inserted into the *Cla*I site of pCGN2016 20 (described in Example 6), producing pCGN1866C. The *Cla*I fragment of pCGN1866C is used to replace the corresponding wild-type *Cla*I fragment of PCGN1867 (described below) to produce pCGN1868. Bce4 coding sequences are removed by digestion of pCGN1868 with *Bam*HI and recircularization of 25 the plasmid to produce pCGN1870. The Bce4 expression cassette, pCGN1870, contains 7.4 kb of 5' regulatory sequence and 1.9 kb of 3' regulatory sequence derived from the Bce4 genomic clone separated by the cloning sites, *Xho*I, *Bam*HI, and *Sma*I. Desaturase sequences in sense or 30 anti-sense orientation may be inserted into the cassette via the cloning sites and the resulting construct may be employed in a plant transformation technique.

pCGN1867

The *Bam*HI and *Sma*I sites of pUC18 are removed by *Bam*HI-*Sma*I digestion and recircularizing of the plasmid, without repair of the ends, to produce pCGN1862. The *Pst*I 5 fragment of pCGN1857, containing the *Bce4* gene, is inserted into the *Pst*I site of pCGN1862 to produce pCGN1867.

Example 9

In this example, the preparation of a napin 1-2 10 expression cassette containing a plant desaturase is described.

*Preparation of Desaturase Clone*

The desaturase cDNA clone from pCGN2754 is prepared 15 and modified as described in Example 8. The *Xho*I fragment from the resulting clone can be subcloned into the napin 1-2 expression cassette, pCGN1808 (described below) at the unique *Xho*I site. This napin 1-2/desaturase expression cassette can then be inserted into a suitable binary 20 vector, transformed into *A. tumefaciens* strain EHA101 in a like manner as described in Example 7.

Alternatively, the desaturase safflower clone may be prepared such that restriction sites flank the translation start and stop sites, as described in Example 8, with the 25 following modification. PCR was carried out according to manufacturer's instructions except for the initial annealing of the oligonucleotides to the template. The reaction mix was heated to 90°C for 5 min, cooled to 37°C over a one hour period, kept at 37°C for 20 min and then 30 subjected to standard PCR cycles. The PCR product was digested with *Pst*I and ligated to pUC8 (Vieira and Messing (1982) *Gene* 19:2359-268) digested with *Pst*I to produce pCGN3220. The *Nco*I/*Sac*I fragment of pCGN3220 containing the pUC8 vector and the 5' and 3' sequences of the 35 safflower desaturase cDNA was gel purified and ligated to the gel-purified cloned *Nco*I/*Sac*I fragment from pCGN1894 (see Example 6). The resulting plasmid pCGN3222 contains safflower desaturase cDNA sequences partially from the cDNA

clone and partially from the PCR. The regions obtained from the PCR were confirmed by DNA sequencing as being identical to the original cloned sequence.

5 *Expression Cassettes*

Napin 1-2 pCGN1808 Expression Cassette

An expression cassette utilizing 5' upstream sequences and 3' downstream sequences obtainable from *B. campestris* napin gene can be constructed as follows.

10 A 2.7 kb *Xho*I fragment of napin 1-2 (Fig. 10 and SEQ ID NO: 29) containing 5' upstream sequences is subcloned into pCGN789 (a pUC based vector the same as pUC119 with the normal polylinker replaced by the synthetic linker - 5'GGAATTCGTCGACAGATCTCTGCAGCTCGAGGGATCCAAGCTT 3', SEQ ID NO: 41, (which represented the polylinker *Eco*RI, *Sal*I, *Bgl*III, *Pst*I, *Xho*I, *Bam*HI, *Hind*III) and results in pCGN940. The majority of the napin coding region of pCGN940 was deleted by digestion with *Sal*I and religation to form pCGN1800. Single-stranded DNA from pCGN1800 was used in an 15 *in vitro* mutagenesis reaction (Adelman et al., *DNA* (1983) 2:183-193) using the synthetic oligonucleotide 5' GCTTGTTCGCCATGGATATCTTCTGTATGTTC 3', SEQ ID NO: 42. This oligonucleotide inserted an *Eco*RV and an *Nco*I restriction site at the junction of the promoter region and the ATG 20 start codon of the napin gene. An appropriate mutant was identified by hybridization to the oligonucleotide used for the mutagenesis and sequence analysis and named pCGN1801.

25 A 1.7 kb promoter fragment was subcloned from pCGN1801 by partial digestion with *Eco*RV and ligation to pCGN786 (a pCGN566 chloramphenicol based vector with the synthetic linker described above in place of the normal polylinker) cut with *Eco*RI and blunted by filling in with DNA Polymerase I Klenow fragment to create pCGN1802.

30 A 2.1 kb *Sal*I fragment of napin 1-2 (Fig. 10 and SEQ ID NO: 29) containing 3' downstream sequences is subcloned into pCGN789 (described above) and results in pCGN941. pCGN941 is digested with *Xho*I and *Hind*III and the resulting approximately 1.6 kb of napin 3' sequences are inserted

into *Xba*I-*Hind*III digested pCGN1802 to result in pCGN1803. In order to remove a 326 nucleotide *Hind*III fragment inserted opposite to its natural orientation, as a result of the fact that there are 2 *Hind*III sites in pCGN1803, the 5 pCGN1803 is digested with *Hind*III and religated. Following religation, a clone is selected which now contains only 1.25 kb of the original 1.6 napin 3' sequence. This clone, pCGN1808 is the napin 1-2 expression cassette and contains 1.725 kb of napin promoter sequences and 1.265 kb of napin 10 3' sequence with the unique cloning sites *Sal*I, *Bgl*II, *Pst*I and *Xba*I in between.

Napin 1-2 pCGN3223 Expression Cassette

Alternatively, pCGN1808 may be modified to contain 15 flanking restriction sites to allow movement of only the expression sequences and not the antibiotic resistance marker to binary vectors such as pCGN1557 (McBride and Summerfelt, *supra*). Synthetic oligonucleotides containing *Kpn*I, *Not*I and *Hind*III restriction sites are annealed and 20 ligated at the unique *Hind*III site of pCGN1808, such that only one *Hind*III site is recovered. The resulting plasmid, pCGN3200 contains unique *Hind*III, *Not*I and *Kpn*I restriction sites at the 3'-end of the napin 3'-regulatory sequences as confirmed by sequence analysis.

25 The majority of the napin expression cassette is subcloned from pCGN3200 by digestion with *Hind*III and *Sac*I and ligation to *Hind*III and *Sac*I digested pIC19R (Marsh, et al. (1984) *Gene* 32:481-485) to make pCGN3212. The extreme 30 5'-sequences of the napin promoter region are reconstructed by PCR using pCGN3200 as a template and two primers flanking the *Sac*I site and the junction of the napin 5'- promoter and the pUC backbone of pCGN3200 from the pCGN1808 construct. The forward primer contains *Cla*I, *Hind*III, *Not*I, and *Kpn*I restriction sites as well as nucleotides 408- 35 423 of the napin 5'-sequence (from the *Eco*RV site) and the reverse primer contains the complement to napin sequences 718-739 which include the unique *Sac*I site in the 5'- promoter. The PCR was performed using a Perkin Elmer/Cetus

thermocycler according to manufacturer's specifications. The PCR fragment is subcloned as a blunt-ended fragment into pUC8 (Vieira and Messing (1982) *Gene* 19:259-268) and digested with *Hinc*II to give pCGN3217. Sequence of 5 pCGN3217 across the napin insert verifies that no improper nucleotides were introduced by PCR. The napin 5'-sequences in pCGN3217 are ligated to the remainder of the napin expression cassette by digestion with *Cla*I and *Sac*I and ligation to pCGN3212 digested with *Cla*I and *Sac*I. The 10 resulting expression cassette pCGN3221, is digested with *Hind*III and the napin expression sequences are gel purified away and ligated to pIC20H (Marsh, *supra*) digested with *Hind*III. The final expression cassette is pCGN3223, which contains in an ampicillin resistant background, essentially 15 identical 1.725 napin 5' and 1.265 3' regulatory sequences as found in pCGN1808. The regulatory regions are flanked with *Hind*III, *Not*I and *Kpn*I restriction sites and unique *Sal*I, *Bgl*III, *Pst*I, and *Xho*I cloning sites are located between the 5' and 3' noncoding regions.

20 Desaturase sequences in sense or anti-sense orientation may be inserted into a napin expression cassette via the cloning sites. The resulting construct may be employed for plant transformation. For example, one of ordinary skill in the art could also use known 25 techniques of gene cloning, mutations, insertion and repair to allow cloning of a napin expression cassette into any suitable binary vector, such as pCGN1557 (described in Example 7) or other similar vectors.

30 *Desaturase Expression*

The coding region of the safflower desaturase contained in pCGN3222 is cloned into the pCGN3223 napin cassette by digestion with *Xho*I and ligation to pCGN3223 digested with *Xho*I and *Sal*I. The resulting plasmid, 35 pCGN3229 is digested with *Asp*718 and inserted in the binary vector pCGN1578 (McBride and Summerfelt (1990) *Plant Mol. Biol.* 14:269-276) at the unique *Asp*718 site. The resulting binary vector is pCGN3231 and contains the safflower

desaturase coding sequences flanked by the napin 5' and 3' regulatory sequences as well as the plant selectable marker construct, 35s/NPTII/tm1.

The resulting binary vector, pCGN3231, is transformed  
5 into *Agrobacterium* and utilized for plant transformation as described in Example 10. For Northern analysis, total RNA is isolated from day 21 and day 28 post-anthesis developing seed from plants transformed with pCGN3231. Five samples were analyzed at day 21 and two at day 28 post-anthesis.  
10 RNA was isolated by the method of Hughes and Galau (*Plant Mol. Biol. Reporter* (1988) 6: 253-257). Northern blot analysis was performed using a labeled 0.8 kb *Bgl*III fragment of pCGN1894 as a probe. Prehybridization and hybridization was at 42°C in 50% formamide, 10X Denhardt's  
15 solution, 5X SSC, 0.1% SDS, 5mM EDTA and 100ug/ml denatured salmon sperm DNA. Filters were washed at 55°C in 0.1 X SSC, 0.1% SDS. Under these conditions, the probe does not hybridize to the endogenous *Brassica* desaturase gene sequences. mRNA complementary to the safflower desaturase  
20 was detected in all the transgenic samples examined. More mRNA was present at day 28 than at day 21 post-anthesis and the highest level of RNA was seen in transgenic 3231-8. The total safflower desaturase mRNA level was estimated to be ~0.01% of the message at day 28 post-anthesis.  
25 Western analysis (see below) gives a preliminary indication of increased protein in one transformant, 3231-8. However, the Western analysis is complicated by two factors: 1. The presence of cross-reacting material at the same molecular weight as expected for the safflower  
30 desaturase. We believe this material is the endogenous *Brassica* desaturase. 2. The analysis of levels of protein expressed is also complicated by the normal developmental increase in the expression of desaturase protein during this time period. If the seeds examined are not at the  
35 precise developmental stage as the control seeds, quantitative differences in the amount of material seen may be simply due to the normal increase in the *Brassica*

desaturase over this time period and not due to the expression of the safflower desaturase.

Western Analysis

5 Soluble protein is extracted from developing seeds of *Brassica* by homogenization with one volume (1ml/gram fresh weight) of buffer containing 20mM potassium phosphate, pH 6.8. The homogenate is clarified by centrifugation at 12,000 x g for 10 minutes. A second centrifugation is  
10 performed if necessary to provide a non-particulate supernatant.

Protein concentration of the extract is measured by the micromethod of Bradford (*Anal. Biochem.* (1976) 72:248-254). Proteins (20-60 $\mu$ g) are separated by denaturing  
15 electrophoresis by the method of Laemmli (*supra*), and are transferred to nitrocellulose membrane by the method of Towbin *et al.* (*Proc. Nat. Acad. Sci.* (1979) 76:4350-4354).

The nitrocellulose membrane is blocked by incubation at room temperature for 15 minutes or at 4°C overnight in  
20 Tris-buffered saline with Tween 20 (Polyoxyethylene sorbitan monolaurate) and "TTBS-milk", (TTBS = 20mM Tris-HCl, 500mM NaCl, 0.1% Tween 20 (v/v), pH 7.5; "TTBS-milk" = TTBS and 3% skim milk powder). The volume of liquid in all  
incubations with the nitrocellulose membrane is sufficient  
25 to cover the membrane completely. The membrane is then  
incubated for an additional 5 minutes in TTBS.

The nitrocellulose membrane is incubated for at least one hour with shaking at room temperature with rabbit anti-stearoyl-ACP desaturase antiserum that was diluted 5,000-  
30 or 10,000-fold in "TTBS-milk". The rabbit anti-desaturase antiserum was commercially prepared from desaturase protein (purified as described in Example 1) by Berkeley Antibody Co. (Richmond, CA). The membrane is washed twice by shaking with TTBS for 5 minutes and then with deionized H<sub>2</sub>O  
35 for 30 seconds.

The nitrocellulose membrane is incubated for at least 45 minutes at room temperature in a solution of "TTBS-milk" in which anti-rabbit IgG-alkaline phosphatase conjugate

(Promega, Madison, WI) is diluted 7,500-fold. The membrane is washed twice in TTBS followed by deionized H<sub>2</sub>O, as described above.

The nitrocellulose membrane is equilibrated in buffer 5 containing 100mM Tris-HCl, 100mM NaCl, 50mM MgCl<sub>2</sub>, pH 9.5, by shaking for 5 minutes. The color reaction is initiated by placing the nitrocellulose membrane into 50ml of the same buffer to which has been added 15mg *p*-nitroblue 10 tetrazolium chloride and 7.5mg 5-bromo- 4 chloro- 3-indolyl phosphate toluidine salt (BioRad Labs; Richmond, CA). The color reaction is stopped by rinsing the nitrocellulose membrane with deionized H<sub>2</sub>O and drying it between filter papers.

Oil analysis of developing seeds indicated no 15 significant change in oil composition of the transformed plants with respect to the control plants. This result is consistant with the low levels of safflower mRNA observed in transgenic plants as compared to levels of endogenous *Brassica* desaturase (Example 12).

20

#### Example 10

In this example, an *Agrobacterium*-mediated plant transformation is described. *Brassica napus* is exemplified. The method is also useful for transformation 25 of other *Brassica* species including *Brassica campestris*.

#### *Plant Material and Transformation*

Seeds of *Brassica napus* cv. Delta are soaked in 95% ethanol for 2 min, surface sterilized in a 1.0% solution of 30 sodium hypochlorite containing a drop of Tween 20 for 45 min., and rinsed three times in sterile, distilled water. Seeds are then plated in Magenta boxes with 1/10th concentration of Murashige minimal organics medium (Gibco) supplemented with pyrodoxine (50 µg/l), nicotinic acid (50 35 µg/l), glycine (200 µg/l), and 0.6% Phytagar (Gibco) pH 5.8. Seeds are germinated in a culture room at 22°C in a 16 h photoperiod with cool fluorescent and red light of

intensity approximately 65  $\mu$ Einstins per square meter per second ( $\mu$ Em $^{-2}$ s $^{-1}$ ).

Hypocotyls are excised from 7 day old seedlings, cut into pieces approximately 4 mm in length, and plated on 5 feeder plates (Horsch et al. 1985). Feeder plates are prepared one day before use by plating 1.0 ml of a tobacco suspension culture onto a petri plate (100x25 mm) containing about 30 ml MS salt base (Carolina Biological) 100 mg/l inositol, 1.3 mg/l thiamine-HCl, 200 mg KH<sub>2</sub>PO<sub>4</sub> 10 with 3% sucrose, 2,4-D (1.0 mg/l), 0.6% Phytagar, and pH adjusted to 5.8 prior to autoclaving (MS0/1/0 medium). A sterile filter paper disc (Whatman 3 mm) is placed on top of the feeder layer prior to use. Tobacco suspension cultures are subcultured weekly by transfer of 10 ml of 15 culture into 100 ml fresh MS medium as described for the feeder plates with 2,4-D (0.2 mg/l), Kinetin (0.1 mg/l). All hypocotyl explants are preincubated on feeder plates for 24 h. at 22°C in continuous light of intensity 30  $\mu$ Em $^{-2}$ s $^{-1}$  to 65  $\mu$ Em $^{-2}$ s $^{-1}$ .

20 Single colonies of *A. tumefaciens* strain EHA101 containing a binary plasmid are transferred to 5 ml MG/L broth and grown overnight at 30°C. Per liter, MG/L broth contains 5g mannitol, 1 g L-glutamic acid or 1.15 g sodium glutamate, 0.25 g KH<sub>2</sub>PO<sub>4</sub>, 0.10 g NaCL, 0.10 g MGSO<sub>4</sub>·7H<sub>2</sub>O, 1 25 mg biotin, 5 g tryptone, and 2.5 g yeast extract, and the broth is adjusted to pH 7.0. Hypocotyl explants are immersed in 7-12 ml MG/L broth with bacteria diluted to 1x10<sup>8</sup> bacteria/ml and after 10-20 min. are placed onto feeder plates. After 48 h of co-incubation with 30 *Agrobacterium*, the hypocotyl explants are transferred to B5 0/1/0 callus induction medium which contains filter sterilized carbenicillin (500 mg/l, added after autoclaving) and kanamycin sulfate (Boehringer Mannheim) at concentrations of 25 mg/l.

35 After 3-7 days in culture at 65  $\mu$ Em $^{-2}$ s $^{-1}$  to 75  $\mu$ Em $^{-2}$ s $^{-1}$  continuous light, callus tissue is visible on the cut surface and the hypocotyl explants are transferred to shoot induction medium, B5BZ (B5 salts and vitamins supplemented

with 3 mg/l benzylaminopurine, 1 mg/l zeatin, 1% sucrose, 0.6% Phytagar and pH adjusted to 5.8). This medium also contains carbenicillin (500 mg/l) and kanamycin sulfate (25 mg/l). Hypocotyl explants are subcultured onto fresh shoot  
5 induction medium every two weeks.

Shoots regenerate from the hypocotyl calli after one to three months. Green shoots at least 1 cm tall are excised from the calli and placed on medium containing B5 salts and vitamins, 1% sucrose, carbenicillin (300 mg/l),  
10 kanamycin sulfate (50 mg/l) and 0.6% Phytagar) and placed in a culture room with conditions as described for seed germination. After 2-4 weeks shoots which remain green are cut at the base and transferred to Magenta boxes containing root induction medium (B5 salts and vitamins, 1% sucrose, 2  
15 mg/l indolebutyric acid, 50 mg/l kanamycin sulfate and 0.6% Phytagar). Green rooted shoots are tested for NPT II activity.

Example 11

20 In this example, a DNA-bombardment plant transformation is described. Peanut transformation is exemplified.

25 DNA sequences of interest may be introduced as expression cassettes, comprising at least a promoter region, a gene of interest, and a termination region, into a plant genome via particle bombardment as described in European Patent Application 332 855 and in co-pending application USSN 07/225,332, filed July 27, 1988.

Briefly, tungsten or gold particles of a size ranging from 0.5 $\mu$ M-3 $\mu$ M are coated with DNA of an expression  
30 cassette. This DNA may be in the form of an aqueous mixture or a dry DNA/particle precipitate.

Tissue used as the target for bombardment may be from cotyledonary explants, shoot meristems, immature leaflets, or anthers.

35 The bombardment of the tissue with the DNA-coated particles is carried out using a Biolistics™ particle gun (Dupont; Wilmington, DE). The particles are placed in the barrel at variable distances ranging from 1cm-14cm from the

barrel mouth. The tissue to be bombarded is placed beneath the stopping plate; testing is performed on the tissue at distances up to 20 cm. At the moment of discharge, the tissue is protected by a nylon net or a combination of 5 nylon nets with mesh ranging from 10 $\mu$ M to 300 $\mu$ M.

Following bombardment, plants may be regenerated following the method of Atreya, et al., (*Plant Science Letters* (1984) 34:379-383). Briefly, embryo axis tissue or cotyledon segments are placed on MS medium (Murashige and 10 Skoog, *Physio. Plant.* (1962) 15:473) (MS plus 2.0 mg.l 6-benzyladenine (BA) for the cotyledon segments) and incubated in the dark for 1 week at 25  $\pm$  2°C and are subsequently transferred to continuous cool white 15 fluorescent light (6.8 W/m<sup>2</sup>). On the 10th day of culture, the plantlets are transferred to pots containing sterile soil, are kept in the shade for 3-5 days and finally moved to greenhouse.

The putative transgenic shoots are rooted. Integration of exogenous DNA into the plant genome may be 20 confirmed by various methods known to those skilled in the art.

#### Example 12

This example describes methods to obtain desaturase 25 cDNA clones from other plant species using the DNA from the *C. tinctorius*  $\Delta$ -9 desaturase clone as the probe.

##### *Isolation of RNA for Northern Analysis*

Poly(A)+ RNA is isolated from *C. tinctorius* embryos 30 collected at 14-17 days post-anthesis and *Simmondsia chinensis* embryos as described in Example 5.

Total RNA is isolated from days 17-18 days post-anthesis *Brassica campestris* embryos by an RNA minipreparation technique (Scherer and Knauf, *Plant Mol. Biol.* (1987) 9:127-134). Total RNA is isolated from *R. communis* immature endosperm of about 14-21 days post-anthesis by a method described by Halling, et al. (*Nucl. Acids Res.* (1985) 13:8019-8033). Total RNA is isolated

from 10 g each of young leaves from *B. campestris*, *B. napus*, and *C. tinctorius*, by extraction of each sample in 5 ml/g tissue of 4 M guanidine thiocyanate buffer as described by Colbert et al. (Proc. Nat. Acad. Sci. (1983) 80:2248-2252). Total RNA is also isolated from immature embryos of *Cuphea hookeriana* by extraction as above in 10 ml/g tissue.

Total RNA is isolated from immature embryos of California bay (*Umbellularia californica*) by an adaptation of the method of Lagrimini et al. (Proc. Nat. Acad. Sci. (1987) 84:7542-7546). Following homogenization in grinding buffer (2.5 ml/g tissue) as described, RNA is precipitated from the aqueous phase by addition of 1/10 volume 3 M sodium acetate and 2 volumes ethanol, followed by freezing at -80°C for 30 minutes and centrifugation at 13,000 x g for 20 minutes. The pellets are washed with 80% ethanol and centrifugation is repeated as above. The pellets are resuspended in water, two volumes of 4 M LiCl are added, and the samples are placed at -20°C overnight. Samples are centrifuged as above and the pellets washed with 80% ethanol. Ethanol precipitation is repeated as above.

Total RNA is further purified from *B. campestris*, *B. napus*, and *C. tinctorius* leaves, and from *C. tinctorius*, *B. campestris*, California bay, and jojoba, and from *R. communis* immature endosperm, by removing polysaccharides on a 0.25 g Sigma Cell 50 cellulose column. The RNA is loaded onto the column in 1 ml of loading buffer (20 mM Tris-HCl pH 7.5, 0.5M NaCl, 1mM EDTA, 0.1% SDS), eluted with loading buffer, and collected in 500 µl fractions. Ethanol is added to the samples to precipitate the RNA. The samples are centrifuged, and the pellets resuspended in sterile distilled water, pooled, and again precipitated in ethanol. The sample is centrifuged, and the resulting RNA is subjected to oligo(dT)-cellulose chromatography to enrich for poly(A)+ RNA as described by Maniatis et al. (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York (1982)). Poly(A)+ RNA is also

purified from total *Cuphea hookeriana* RNA by oligo(dT)-cellulose chromatography.

*Northern Analysis Using C. tinctorius Desaturase*

Clone: 2.5 µg of poly(A)+ RNA from each of the above

5 described poly(A)+ samples from immature embryos of jojoba, *Cuphea hookeriana*, California bay, *Brassica campestris*, and *C. tinctorius*, from immature endosperm of *R. communis*, and from leaves of *C. tinctorius*, *B. campestris*, and *B. napus* are electrophoresed on formaldehyde/agarose gels (Fourney 10 et al., *Focus* (1988) 10:5-7) and transferred to a Hybond-C extra (Amersham, Arlington Heights, IL) filter according to manufacturer's specifications. The filter is prehybridized for four hours and hybridized overnight at 42°C in a roller bottle containing 10 ml of hybridization buffer (1 M NaCl, 15 1% SDS, 50% formamide, 0.1 mg/ml denatured salmon sperm DNA) in a Hybridization Incubator, model 1040-00-1 (Robbins Scientific Corporation, Sunnyvale, CA). The probe used in the hybridization is a gel-isolated *Bgl*II fragment of the Δ-9 desaturase clone that is labeled with <sup>32</sup>P-dCTP using a 20 BRL (Gaithersburg, MD) nick-translation kit, following manufacturer's instructions. The blot is washed three times for 20 minutes each in 2X SSC, 0.5% SDS at 55°C. The blot is exposed at -80°C, with a Dupont Cronex intensifying screen, to X-ray film for four days.

25 The autoradiograph shows that the *C. tinctorius* desaturase gene is expressed in both immature embryos and leaves of *C. tinctorius*, although the level of expression is considerably higher in embryos than in leaves. The autoradiograph also shows hybridization of the *C. 30 tinctorius* desaturase clone to mRNA bands of a similar size in immature embryos from jojoba and California bay, and immature endosperm from *R. communis*. Hybridization is also detectable in RNA from *B. campestris* embryos upon longer exposure of the filter to X-ray film.

35 *R. communis* cDNA Library Construction: A plant seed cDNA library may be constructed from poly(A)+ RNA isolated from *R. communis* immature endosperm as described above. The plasmid cloning vector PCGN1703, and cloning method are

as described in Example 5. The *R. communis* endosperm cDNA bank contains approximately  $2 \times 10^6$  clones with an average cDNA insert size of approximately 1000 base pairs.

The *R. communis* immature endosperm cDNA bank is moved  
5 into the cloning vector lambda gt22 (Stratagene Cloning  
Systems) by digestion of total cDNA with *NotI* and ligation  
to lambda gt22 DNA digested with *NotI*. The resulting phage  
are packaged using a commercially available kit and titered  
10 using *E. coli* strain LE392 (Stratagene Cloning Systems, La  
Jolla, CA). The titer of the resulting library was  
approximately  $1.5 \times 10^7$  pfu/ml.

*R. communis* cDNA Library Screen: The library is  
plated on *E. coli* strain LE392 at a density of  
approximately 25,000 pfu/150mm NZY plate to provide  
15 approximately 50,000 plaques for screening. Phage are  
lifted in duplicate on to NEN (Boston, MA.) Colony/Plaque  
Screen filters as described in Example 5. Following  
prehybridization at 42°C in 25 ml of hybridization buffer  
(1 M NaCl, 1% SDS, 50% formamide, 0.1 mg/ml denatured  
20 salmon sperm DNA) filters are hybridized overnight with a  
gel-purified 520 base pair *Bgl*III fragment of the *C.*  
*tinctorius* desaturase clone (Figure 7A) that is  
radiolabeled with  $^{32}\text{P}$ -dCTP using a BRL (Gaithersburg, MD)  
Nick Translation System. Filters are washed three times  
25 for 20 minutes each in 2X SSC, 0.5% SDS at 55°C in a  
shaking water bath. Filters are exposed to X-ray film  
overnight at -80°C with a Dupont Cronex intensifying  
screen.

Clones are detected by hybridization on duplicate  
30 filters with the *C. tinctorius* desaturase cDNA fragment and  
plaque purified. During plaque purification, it was  
observed that larger plaques were obtained when *E. coli*  
strain Y1090 (Young, R.A. and Davis, R.W., *Proc. Natl.*  
*Acad. Sci. USA* (1983) 80:1194) was used as the host  
35 strain. This strain was thus used in subsequent plaque  
purification steps. Phage DNA is prepared from the  
purified clones as described by Grossberger (*NAR* (1987)  
15:6737) with the following modification. The proteinase K

treatment is replaced by the addition of 10% SDS and a 10 minute incubation at room temperature. Recovered phage DNA is digested with *EcoRI*, religated at low concentration, and transformed into *E. coli* DH5 $\alpha$ (BRL; Gaithersburg, MD) cells

5 to recover plasmids containing cDNA inserts in pCGN1703. Minipreparation DNA (Maniatis et al., *supra*) is prepared from the clones and DNA sequence is determined as described above. Partial nucleotide sequence of the cDNA insert of a *R. communis* desaturase clone pCGN3230 is presented in

10 Figure 3A and SEQ ID NO: 14. The complete nucleotide sequence of this clone is presented in Fig. 3B and as SEQ ID NO: 15.

*Northern Analysis Using R. communis Desaturase Clone:*  
Total RNA for Northern analysis is isolated from tobacco

15 leaves by the method of Ursin et al. (*Plant Cell* (1989) 1:727-736), petunia and tomato leaves by the method of Ecker and Davis (*Proc.Nat.Acad.Sci.* (1987) 84:5202-5206), and corn leaves by the method of Turpen and Griffith (*Biotechniques* (1986) 4:11-15). Total RNA samples from

20 tobacco, corn, and tomato leaves are enriched for poly(A)+ RNA by oligo(dT)-cellulose chromatography as described by Maniatis et al. (*supra*).

Poly(A)+ RNA samples from tomato leaves (4  $\mu$ g) and corn and tobacco leaves (1  $\mu$ g each), and total RNA from

25 petunia leaves (25  $\mu$ g) are electrophoresed on a formaldehyde/agarose gel as described by Shewmaker et al. (*Virology* (1985) 140:281-288). Also electrophoresed on this gel are poly(A)+ RNA samples isolated from *B. campestris* day 17-19 embryos and *B. campestris* leaves (2  $\mu$ g each), immature embryos from *C. tinctorius*, bay, and jojoba (1  $\mu$ g each), and *R. communis* endosperm (1  $\mu$ g). The

30 isolation of these poly(A)+ RNA samples is described above for the Northern analysis using *C. tinctorius* desaturase cDNA as probe. The RNA is transferred to a nitrocellulose

35 filter as described by Shewmaker et al. (*supra*) and prehybridized and hybridized at 42°C in 50% formamide, 10X Denhardt's solution (described in Maniatis et al. (*supra*)), 5X SSC, 0.1% SDS, 5 mM EDTA, 100 ug/ml denatured salmon

sperm DNA, and 10% dextran sulfate (in hybridization buffer only). The probe for hybridization is the  $^{32}\text{P}$ -labeled (BRL Nick Translation Kit) 1.7 kb SalI insert of pCGN3230 that has been gel-purified from minipreparation DNA. The filter 5 is washed following hybridization for 30 minutes in 2X SSC, 0.1% SDS at 42°C and at 50°C twice for 15 minutes each. The filter is exposed to X-ray film overnight at -80°C with a Dupont Cronex intensifying screen.

The autoradiograph shows hybridization of the *R. 10 communis* desaturase clone to mRNA bands of a similar size in immature embryos from *B. campestris*, California bay, and *C. tinctorius*, and also in corn leaves and *R. communis* endosperm.

*B. campestris* Embryo cDNA Library Construction: Total 15 RNA is isolated from 5 g of *B. campestris* cv. R500 embryos obtained from seeds harvested at days 17-19 post-anthesis. RNA is extracted in 25 mls of 4 M guanidine thiocyanate buffer as described by Colbert et al. (PNAS (1983) 80:2248-2252). Polysaccharides are removed from the RNA sample by 20 resuspending the pellet in 6 ml of 1X TE (10 mM Tris/1 mM EDTA pH 8), adding potassium acetate to a concentration of 0.05M, and adding one half volume of ethanol. The sample is placed on ice for 60 minutes and centrifuged for 10 minutes at 3000 x g. RNA is precipitated from the supernatant by 25 adding sodium acetate to a concentration of 0.3 M followed by the addition of two volumes of ethanol. RNA is recovered from the sample by centrifugation at 12,000 x g for 10 minutes and yield calculated by UV spectrophotometry. Two mg of the total RNA is further purified by removing 30 polysaccharides on a 0.25 g Sigma Cell 50 cellulose column, as described above, and is also enriched for poly(A)+ RNA by oligo(dT)-cellulose chromatography as described above.

A *B. campestris* day 17-19 post anthesis embryo cDNA library is constructed in plasmid vector pCGN1703 as 35 described in Example 5, using 5 ug of the above described poly(A)+ RNA. The library, which consists of approximately  $1.5 \times 10^5$  transformants, is amplified by plating and scraping colonies, and is stored as frozen *E. coli* cells in

10% DMSO at -80° C. DNA is isolated from a portion of the amplified library by scaling up the alkaline lysis technique of Birnboim and Doly (*Nucleic Acids Res.* (1979) 7:1513), and purified by CsCl centrifugation. Library DNA 5 is digested with *EcoRI* and is cloned into *EcoRI*-digested bacteriophage lambda gt10 (Stratagene; La Jolla, CA) DNA. The DNA is packaged using Gigapack II Gold *in vitro* packaging extracts (Stratagene; La Jolla, CA) according to manufacturer's specifications. The titer of the phage 10 stock, determined by dilution plating of phage in *E. coli* C600 hfl- cells (Huynh, et al., *DNA Cloning. Volume 1*. Eds. Gover, D.M. (1985) IRL Press Limited: Oxford, England, pp. 56,110), is  $6 \times 10^6$  pfu per ml.

*B. campestris* cDNA Library Screen: The library is 15 plated on *E. coli* strain C600 hfl- at a density of approximately 30,000 pfu/150mm NZY plate to provide approximately 120,000 plaques for screening. Phage are lifted in duplicate on to NEN (Boston, MA.) Colony/Plaque Screen filters as described in Example 5. Filters are 20 prehybridized and hybridized with the  $^{32}\text{P}$ -labeled fragment of pCGN3230 as described above for the Northern hybridization. Filters are washed for 30 minutes in 2X SSC, 0.1% SDS at 50°C and at 55°C twice for 15 minutes each. Filters are exposed to X-ray film overnight at -80°C 25 with a Dupont Cronex intensifying screen.

Clones are detected by hybridization on duplicate filters to the *R. communis* desaturase cDNA fragment and plaque purified. During plaque purification, the probe used was a gel-purified 1.4 kb *SstI* fragment of pCGN3230 30 which lacks the poly(A)+ tail. As described above, phage DNA is isolated from purified lambda clones, digested with *EcoRI*, ligated, and transformed to *E. coli* DH5 $\alpha$  cells. Minipreparation DNA is prepared and partial DNA sequence determined as described above. Partial DNA sequences of 35 two clones, pCGN3235 and pCGN3236, are presented in Figure 4A (SEQ ID NO: 17) and 4B (SEQ ID NO: 18), respectively. Initial DNA sequence analysis of the 3' regions of these clones indicates that pCGN3236 and pCGN3235 are cDNA

clones from the same gene. pCGN3236 is a shorter clone than pCGN3235, which appears to contain the entire coding region of the *B. campestris* desaturase gene. The complete nucleotide sequence of pCGN3235 is presented in Figure 4C  
5 and SEQ ID NO: 19.

Desaturase Gene Analysis: Southern and Northern analyses of *Brassica* species are conducted to determine the number of genes which encode the *Brassica* desaturase clone, pCGN3235 in *B. campestris*, *B. oleracea*, and *B. napus*, and  
10 the timing of expression of the gene in *B. campestris* developing seeds. DNA is isolated from leaves of each of the above-named *Brassica* species by the method of Bernatzky and Tanksley (*Theor. Appl. Genet.* (1986) 72:314-321). DNA from each of the species is digested with restriction  
15 endonucleases EcoRI and XbaI (10 ug/digest), electrophoresed in a 0.7% agarose gel, and transferred to a nitrocellulose filter (Maniatis et al., *supra*). The filter is prehybridized and hybridized at 42°C (as described above for Northern analysis using *R. communis* desaturase clone)  
20 with a <sup>32</sup>P-labeled (nick translation) gel-isolated HindIII/PvuII fragment of pCGN3235 (Fig. 7C). The filter is washed following overnight hybridization, for 30 minutes at 55°C in 1X SSC, 0.1% SDS, followed by two 15 minute washed at 55°C in 0.1X SSC, 0.1% SDS.

25 The autoradiograph indicates that the *Brassica* desaturase is encoded by a small gene family consisting of about two genes in *B. campestris* and *B. oleracea*, and about four genes in *B. napus*.

The timing of expression of the desaturase gene during  
30 seed development is determined by Northern analysis. RNA is isolated from immature seeds of *B. campestris* cv. R500 collected at 11, 13, 15, 17, 19, 21, 25, 30, 35, and 40 days post-anthesis. Total RNA is isolated as described by Scherer and Knauf (*Plant Mol. Biol.* (1987) 9:127-134).  
35 Twenty five micrograms of RNA from each time point are electrophoresed through a formaldehyde-containing 1.5% agarose gel as described by Shewmaker, et al. (*supra*) and blotted to nitrocellulose (Thomas, *Proc. Nat. Acad. Sci.*

(1980) 77:5201-5205). The blot is pre-hybridized and hybridized at 42°C with the  $^{32}\text{P}$ -labeled HindIII/PvuII fragment of pCGN3235 as described above. The filter is washed following overnight hybridization, for 30 minutes at 5 55°C in 1X SSC, 0.1% SDS, followed by two 15 minute washed at 55°C in 0.1X SSC, 0.1% SDS.

10 The autoradiograph indicates that the desaturase gene is expressed in *B. campestris* developing seeds beginning at about day 19 and through about day 30, with maximal expression at day 25. By a similar Northern analysis, the level of desaturase mRNA in developing *Brassica napus* seeds (day 21) was estimated to be approximately 1% of the total mRNA.

15 *Isolation of Other Desaturase Gene Sequences:* cDNA libraries may be constructed as described above and genomic libraries can be constructed from DNA from various sources using commercially available vectors and published DNA isolation, fractionation, and cloning procedures. For example, a *B. campestris* genomic library can be constructed 20 using DNA isolated according to Scofield and Crouch (J.Biol.Chem. (1987) 262:12202-12208) that is digested with BamHI and fractionated on sucrose gradients (Maniatis et al., *supra*), and cloned into the lambda phage vector LambdaGem-11 (Promega; Madison, WI) using cloning procedures 25 of Maniatis et al. (*supra*).

30 cDNA and genomic libraries can be screened for desaturase cDNA and genomic clones, respectively, using published hybridization techniques. Screening techniques are described above for screening libraries with DNA fragments. Libraries may also be screened with synthetic oligonucleotides, for example using methods described by Berent et al. (BioTechniques (1985) 3:208-220). Probes for the library screening can be prepared by PCR, or from the sequences of the desaturase clones provided herein. 35 Oligonucleotides prepared from the desaturase sequences may be used, as well as longer DNA fragments, up to the entire desaturase clone.

For example, jojoba polyadenylated RNA is used to construct a cDNA library in the cloning vector  $\lambda$ ZAPII/EcoRI (Stratagene, San Diego, CA). RNA is isolated from jojoba embryos collected at 80-90 days post-anthesis by isolating 5 polyribosomes using a method initially described by Jackson and Larkins (Plant Physiol. (1976) 57:5-10) and modified by Goldberg et al. (Developmental Biol. (1981) 83:201-217). Polysaccharide contaminants in the polyribosomal RNA preparation are removed by running the RNA over a cellulose 10 column (Sigma-cell 50) in high salt buffer (0.5M NaCl, 20mM Tris pH 7.5, 1mM EDTA, 0.1% SDS). The contaminant binds to the column and the RNA is collected in the eluant. The eluant fractions are pooled and the RNA is ethanol 15 precipitated. The precipitated total RNA is then resuspended in a smaller volume and applied to an oligo d(T) cellulose column to isolate the polyadenylated RNA.

The library is constructed using protocols, DNA and bacterial strains as supplied by the manufacturer. Clones are packaged using Gigapack Gold packaging extracts 20 (Stratagene), also according to manufacturer's recommendations. The cDNA library constructed in this manner contins approximately  $1 \times 10^6$  clones with an average cDNA insert size of approximately 400 base pairs.

The jojoba library is plated on *E. coli* XL1-Blue 25 (Stratagene) at a density of approximately 5000pfu/150mm plate to provide approximately 60,000 plaques for screening. Phage are lifted onto duplicate nylon membrane filters as described previously. Filters are prehybridized at 42°C in a hybridization buffer containing 40% formamide, 30 10X Denhardt's solution, 5X SSC, 0.1% SDS, 50mM EDTA, and 100 $\mu$ g/ml denatured salmon sperm DNA. Hybridization is at 42°C in the same buffer with added nick translated (BRL 35 Nick Translation System) 520 bp *Bgl*II fragment of the *C. tinctorius* desaturase clone described previously. Filters are washed at 50°C in 2X SSC and exposed to X-ray film overnight.

Desaturase clones are detected by hybridization on duplicate filters with the *C. tinctorius* cDNA fragment and

plaque-purified. Positive clones are recovered as plasmids in *E. coli* following manufacturer's directions and materials for *in vivo* excision. Partial, preliminary DNA sequence of a clone, 3-1, is determined and the 5 corresponding amino acid sequence is translated in three frames. In this manner, homology to the *C. tinctorius* desaturase cDNA clone is detected in one reading frame. The preliminary DNA sequence of this jojoba desaturase cDNA fragment is shown in Figure 5 (SEQ ID NO: 43). Also shown 10 is the corresponding translated amino acid sequence in the reading frame having *C. tinctorius* desaturase homology. The jojoba cDNA fragment is approximately 75% homologous at the DNA level and approximately 79% homologous at the amino acid level compared to sequence of the *C. tinctorius* 15 desaturase in this region.

Example 13

Antisense constructs are described which allow for transcription of a reverse copy of the *B. campestris* 20 desaturase cDNA clone in the 5' to 3' orientation of transcription.

*Preferential Expression of Antisense Constructs in Embryos*

In order to reduce the transcription of a desaturase 25 gene in embryos of *B. napus* or *B. campestris*, constructs may be prepared which allow for production of antisense copies of the desaturase cDNA preferentially in the embryos. Promoter sequences which are desirable to obtain this pattern of expression include, but are not limited to, 30 the ACP, Bce4, and napin 1-2 expression cassettes described in Examples 7, 8, and 9, respectively. It also may be desirable to control the expression of reverse copies of the desaturase cDNA under two different promoters in the same transformed plant to provide for a broader timing of 35 expression of the antisense desaturase DNA. For example, expression from the ACP promoter may begin and end earlier than expression from the napin promoter. Thus, expressing the reverse desaturase from both promoters may result in

the production of the antisense strand of DNA over a longer period of embryo development.

An example of expression of an antisense desaturase gene preferentially in the embryos is provided below.

5 Similar constructs containing the same or a different fragment of the desaturase gene and any of the promoters described above, as well as other promoter regions which may be useful, may also be prepared using gene cloning, insertion, mutation and repair techniques well known to  
10 those of ordinary skill in the art.

A. Antisense Desaturase Expression from the ACP Promoter

Construction of pCGN3239 is as follows:

pCGN3235 (Example 12) is digested with *Pvu*II and  
15 *Hind*III and the *Hind*III sticky ends are filled in with Klenow in the presence of 200  $\mu$ M dNTPs. The 1.2 kb *Pvu*II/*Hind*III fragment containing the desaturase coding sequence is gel purified and ligated in the antisense orientation into *Eco*RV-digested pCGN1977 (ACP expression  
20 cassette; described in Example 7) to create pCGN3238. The 4.2 kb *Xba*I/*Asp*718 fragment of pCGN3238 containing the antisense desaturase in the ACP cassette is transferred into *Xba*I/*Asp*718-digested pCGN1557 (binary transformation vector; described in Example 7) to create pCGN3239.

25 B. Antisense Desaturase Expression From The Napin Promoter

Construction of pCGN3240 is as follows: pCGN3235 is digested with *Pvu*II and *Hind*III, the sticky ends are blunted, and the resulting fragment is inserted in an anti-  
30 sense orientation into pCGN3223 which has been digested with *Sal*I and blunted with Klenow enzyme. The resulting plasmid, pCGN3240 will express an anti-sense desaturase RNA from the napin promoter cassette.

35 C. Antisense Desaturase Expression From a Dual Promoter Cassette

Construction of pCGN3242 is as follows: An *Asp*718 fragment of pCGN3240 containing the napin 5' and 3' regions surrounding the desaturase sequences is inserted into the

Asp718 site of pCGN3239 (a binary vector containing an ACP promoter, antisense desaturase construct) to create pCGN3242.

5 *Constitutive Transcription*

A. Binary Vector Construction

1. Construction of pCGP291.

The *Kpn*I, *Bam*HI, and *Xba*I sites of binary vector pCGN1559 (McBride and Summerfelt, *Pl.Mol.Biol.* (1990) 14: 10 269-276) are removed by *Asp*718/*Xba*I digestion followed by blunting the ends and recircularization to produce pCGP67. The 1.84 kb *Pst*I/*Hind*III fragment of pCGN986 containing the 35S promoter-tml3' cassette is inserted into *Pst*I/*Hind*III digested pCGP67 to produce pCGP291.

15 2. Construction of pCGN986.

The 35S promoter-tml3' expression cassette, pCGN986, contains a cauliflower mosaic virus 35S (CaMV35) promoter and a T-DNA tml 3'-region with multiple restriction sites between them. pCGN986 is derived from another cassette, pCGN206, containing a CaMV35S promoter and a different 3' region, the CaMV region VI 3'-end. The CaMV 35S promoter is cloned as an *Alu*I fragment (bp 7144-7734) (Gardner et. al., *Nucl.Acids Res.* (1981) 9:2871-2888) into the *Hinc*II site of M13mp7 (Messing, et. al., *Nucl.Acids Res.* (1981) 25 9:309-321) to create C614. An *Eco*RI digest of C614 produced the *Eco*RI fragment from C614 containing the 35S promoter which is cloned into the *Eco*RI site of pUC8 (Vieira and Messing, *Gene* (1982) 19:259) to produce pCGN147.

30 pCGN148a containing a promoter region, selectable marker (KAN with 2 ATG's) and 3' region, is prepared by digesting pCGN528 with *Bgl*II and inserting the *Bam*HI-*Bgl*II promoter fragment from pCGN147. This fragment is cloned into the *Bgl*II site of pCGN528 so that the *Bgl*II site is proximal to the kanamycin gene of pCGN528.

35 The shuttle vector used for this construct, pCGN528, is made as follows: pCGN525 is made by digesting a plasmid containing Tn5 which harbors a kanamycin gene (Jorgenson

et. al., *Mol. Gen. Genet.* (1979) 177:65) with *HindIII-BamHI* and inserting the *HindIII-BamHI* fragment containing the kanamycin gene into the *HindIII-BamHI* sites in the tetracycline gene of pACYC184 (Chang and Cohen, J. 5 *Bacteriol.* (1978) 134:1141-1156). pCGN526 was made by inserting the *BamHI* fragment 19 of pTiA6 (Thomashow et. al., *Cell* (1980) 19:729-739), modified with *XbaI* linkers inserted into the *SmaI* site, into the *BamHI* site of pCGN525. pCGN528 is obtained by deleting the small *XbaI* 10 fragment from pCGN526 by digesting with *XbaI* and religating.

pCGN149a is made by cloning the *BamHI*-kanamycin gene fragment from pMB9KanXXI into the *BamHI* site of pCGN148a. pMB9KanXXI is a pUC4K variant (Vieira and Messing, *Gene* 15 (1982) 19:259-268) which has the *XbaI* site missing, but contains a functional kanamycin gene from Tn903 to allow for efficient selection in *Agrobacterium*.

pCGN149a is digested with *HindIII* and *BamHI* and ligated to pUC8 digested with *HindIII* and *BamHI* to produce 20 pCGN169. This removes the Tn903 kanamycin marker. pCGN565 and pCGN169 are both digested with *HindIII* and *PstI* and ligated to form pCGN203, a plasmid containing the CaMV 35S promoter and part of the 5'-end of the Tn5 kanamycin gene (up to the *PstI* site, Jorgenson et. al., (1979), *supra*). A 25 3'-regulatory region is added to pCGN203 from pCGN204, an *EcoRI* fragment of CaMV (bp 408-6105) containing the region VI 3' cloned into pUC18 (Yanisch-Perron, et al., *Gene* (1985) 33:103-119) by digestion with *HindIII* and *PstI* and ligation. The resulting cassette, pCGN206, is the basis 30 for the construction of pCGN986.

The pTiA6 T-DNA tml 3'-sequences are subcloned from the *Bam*19 T-DNA fragment (Thomashow et al., (1980) *supra*) as a *BamHI-EcoRI* fragment (nucleotides 9062 to 12,823, numbering as in Barker et al., *Plant Mol. Biol.* (1982) 35 2:335-350) and combined with the pACYC184 (Chang and Cohen (1978), *supra*) origin of replication as an *EcoRI-HindIII* fragment and a gentamycin resistance marker (from plasmid

pLB41), obtained from D. Figurski) as a *Bam*HI-*Hind*III fragment to produce pCGN417.

The unique *Sma*I site of pCGN417 (nucleotide 11,207 of the *Bam*19 fragment) is changed to a *Sac*I site using linkers 5 and the *Bam*HI-*Sac*I fragment is subcloned into pCGN565 to give pCGN971. The *Bam*HI site of pCGN971 is changed to an *Eco*RI site using linkers. The resulting *Eco*RI-*Sac*I fragment containing the *tml* 3' regulatory sequences is joined to pCGN206 by digestion with *Eco*RI and *Sac*I to give 10 pCGN975. The small part of the *Tn*5 kanamycin resistance gene is deleted from the 3'-end of the *CaMV* 35S promoter by digestion with *Sal*I and *Bgl*III, blunting the ends and ligation with *Sal*I linkers. The final expression cassette pCGN986 contains the *CaMV* 35S promoter followed by two *Sal*I 15 sites, an *Xba*I site, *Bam*HI, *Sma*I, *Kpn*I and the *tml* 3' region (nucleotides 11207-9023 of the T-DNA).

B. Insertion of Desaturase Sequence

The 1.6 kb *Xba*I fragment from pCGN3235 containing the desaturase cDNA is inserted in the antisense orientation 20 into the *Xba*I site of pCGP291 to produce pCGN3234.

*Plant Transformation*

The binary vectors containing the expression cassette and the desaturase gene are transformed into *Agrobacterium* 25 *tumefaciens* strain EHA101 (Hood, et al., *J. Bacteriol.* (1986) 168:1291-1301) as per the method of Holsters, et al., *Mol. Gen. Genet.* (1978) 163:181-187. Transformed *B. napus* and/or *Brassica campestris* plants are obtained as described in Example 10.

30

*Analysis of Transgenic Plants*

A. Analysis of pCGN3242 Transformed *Brassica campestris* cv. Tobin Plants

35 Due to the self-incompatibility of *Brassica campestris* cv. Tobin, individual transgenic plants are pollinated using non-transformed Tobin pollen. Because of this, the T2 seeds of a transgenic plant containing the antisense desaturase at one locus would be expected to segregate in a

1:1 ratio of transformed to non-transformed seed. The oil composition of ten individual seeds collected at 26 days post-anthesis from several pCGN3242-transformed plants and one non-transformed control was analyzed by gas chromatography according to the method of Browse, et al., *Anal. Biochem.* (1986) 152:141-145. One transformant, 3242-T-1, exhibits an oil composition that differed distinctly from controls on preliminary analysis. The control Tobin seeds contained an average of 1.8% 18:0 (range 1.5% - 2.0%) and 52.9% 18:1 (range 48.2% - 57.1%). T2 seeds of 3242-T-1 segregated into two distinct classes. Five seeds contained levels of 18:0 ranging from 1.3% to 1.9% and levels of 18:1 ranging from 42.2% to 58.3%. The other five seeds contained from 22.9% to 26.3% 18:0 and from 19.9% to 26.1% 18:1.

**B. Analysis of pCGN3234 Transformed Plants**

Some abnormalities have been observed in some transgenic *Brassica napus* cv. Delta and Bingo and *Brassica campestris* cv. Tobin plants containing pCGN3234. These effects could be due to the constitutive expression of antisense desaturase RNA from the 35S promoter or could be due to the transformation/tissue culture regime the plants have been subjected to.

The above results demonstrate the ability to obtain plant  $\Delta$ -9 desaturases, isolate DNA sequences which encode desaturase activity and manipulate them. In this way, the production of transcription cassettes, including expression cassettes can be produced which allow for production, including specially differentiated cell production of the desired product. A purified *C. tinctorius* desaturase is provided and used to obtain nucleic acid sequences of *C. tinctorius* desaturase. Other plant desaturase sequences are provided such as *R. communis*, *B. campestris*, and *S. chinensis*. These sequences as well as desaturase sequences obtained from them may be used to obtain additional desaturase, and so on. And, as described in the application modification of oil composition may be achieved.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains.

5 All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

10 Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claim.

15

What is claimed is:

1. A recombinant DNA construct comprising a sequence encoding at least a portion of a plant desaturase, said desaturase when mature having activity toward an unsaturated fatty acid substrate.
2. The construct of Claim 1 encoding a biologically active plant desaturase.
3. The construct of Claim 1 wherein said sequence encodes a precursor desaturase.
- 10 4. The construct of Claim 1 wherein said sequence encodes a mature desaturase.
5. The construct of Claim 1 wherein said sequence encodes a transit peptide.
- 15 6. The construct of Claim 1 comprising a cDNA sequence.
7. The construct of Claim 1 wherein said sequence is joined to a second nucleic acid sequence which is not naturally joined to said first sequence.
- 20 8. The construct of Claim 1 comprising, in the 5' to 3' direction of transcription, a transcriptional regulatory region functional in a host cell and said sequence.
- 25 9. The construct of Claim 8 further comprising, a translational regulatory region immediately 5' to said sequence and a transcriptional/translational termination regulatory region 3' to said sequence, wherein said regulatory regions are functional in said host cell.
10. The construct of Claim 8 wherein said sequence is a sense sequence.
- 30 11. The construct of Claim 8 wherein said sequence is an anti-sense sequence.
12. The construct of Claim 8 wherein said host cell is a plant cell.
- 35 13. The construct of Claim 12 wherein said transcriptional initiation region is obtained from a gene preferentially expressed in plant seed tissue during lipid accumulation.
14. The construct of Claim 13 wherein said transcriptional initiation region is selected from the

regulatory region 5' upstream to a structural gene of the group consisting of any one of *Bce4*, *seed ACP Bcg 4-4* and *napin 1-2*.

15. The construct of Claim 9 wherein said  
5 transcriptional termination region is a plant desaturase  
termination region.

16. The construct of Claim 1 wherein said plant  
desaturase is a  $\Delta$ -9 desaturase.

17. The construct of Claim 1 wherein said sequence is  
10 obtainable from any one of *C. tinctorius*, *R. communis* and  
*B. campestris*.

18. A method of modifying fatty acid composition in a  
plant host cell from a given percentage of fatty acid  
saturation to a different percentage of fatty acid  
15 saturation comprising

growing a host plant cell having integrated into its  
genome a recombinant DNA sequence encoding a plant  
desaturase under the control of regulatory elements  
functional in said plant cell during lipid accumulation  
20 under conditions which will promote the activity of said  
regulatory elements.

19. The method of Claim 18 wherein the overexpression  
of plant desaturase is obtained.

20. The method of Claim 18 wherein the decrease of  
25 endogenous plant desaturase is obtained.

21. The method of Claim 18 wherein said regulatory  
elements function preferentially in plant seed.

22. The method of Claim 20 wherein the percentage of  
long chain unsaturated fatty acids is increased.

30 23. A plant cell having a modified level of saturated  
fatty acids produced according to the method of any one of  
Claims 18-22.

24. The plant cell of Claim 23 wherein said cell is a  
*Brassica* plant cell.

35 25. The plant cell of Claim 23 wherein said cell is  
*in vivo*.

26. The plant cell of Claim 23 wherein said cell is  
an oilseed embryo plant cell.

27. A plant seed having a modified level of saturated fatty acids as compared to a seed of said plant having a native level of saturated fatty acids produced according to a method comprising

5 growing a plant, having integrated into the genome of embryo cells a recombinant DNA sequence encoding a plant desaturase under the control of regulatory elements functional in seed during lipid accumulation, to produce seed under conditions which will promote the activity of  
10 said regulatory elements, and  
harvesting said seed.

28. The seed of Claim 27 wherein said plant is *Brassica napus*.

29. The seed of claim 27 wherein said seed is an  
15 oilseed.

30. The seed of Claim 27 wherein said plant desaturase is a  $\Delta$ -9 desaturase.

31. A plant seed oil of a plant having an endogenous level of saturated fatty acids comprising a plant seed oil  
20 having a modified level of saturated fatty acids.

32. The oil of Claim 31 comprising a *Brassica napus* oil.

33. A plant seed oil separated from an seed produced according to any one of Claims 27-30.

25 34. A host cell comprising a plant desaturase encoding sequence of any one of Claims 1-17.

35. The cell of Claim 34 wherein said cell is a plant cell.

30 36. The cell of Claim 35 wherein said plant cell is *in vivo*.

37. The cell of Claim 35 wherein said plant cell is a *Brassica* plant cell.

38. A transgenic host cell comprising an expressed plant desaturase.

35 39. The cell of Claim 38 wherein said host cell is a plant cell.

40. The cell of Claim 38 wherein said plant desaturase is a  $\Delta$ -9 desaturase.

41. A method of producing a plant desaturase in a host cell or progeny thereof comprising growing a host cell or progeny thereof comprising a construct of any one of Claims 1-10 and 12-17 under 5 conditions which will permit the production of said plant desaturase.

42. The method of Claim 41 wherein said host cell is a plant cell and said construct is integrated into the genome of said plant cell.

10 43. The method of Claim 42 wherein said plant cell is *in vivo*.

44. A host cell comprising a plant desaturase produced according to Claim 41.

15 45. The cell of Claim 45 wherein said host cell is a plant host cell and said construct is integrated into the genome of said plant cell.

F1:	ASTLGSSSTPKVDNAKKPFQQPREVHVQVTHS <sub>X</sub> MPPQKIEIFKSIEG <sub>W</sub> RAEQNIIIV <sub>F</sub> <sup>H</sup> LKPVEKCWQ
F2:	DFLPDPAS <sub>T</sub> EGFDEQVKELRARAKEIPDDYFVVVLVQDMITEALPTYOTMLNTLDGV
F3:	DETGASLTPWAVWT
F4:	DLLHTYL <sub>L</sub> SGRV
F5:	DMRQIQKTIQYLI
F6:	TENSPYLGFIYT <sub>S</sub> FQER
F7:	DVFLAQI <sub>Q</sub> GTIASDEKRHETAYTKIVEKLFIELDPDGTVLAFA <sub>T</sub> ADMMRKKI <sub>T</sub> S <sub>M</sub> PAHLMY
F8:	DNLF
F9:	dvF1AV <sub>T</sub> <sup>A</sup> QRL <sub>I</sub> <sup>G</sup> VYVTAK
F10:	DYADILEFLVGRWK
F11:	VADLTGLSGEGRKA <sub>G</sub> <sup>O</sup> DYVCGLPPRIRRLEERAQGRAKEGPVVVF <sub>S</sub> WIFDRQVKL

FIGURE 1

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HindIII   1 GCTCACCTGGTGGAGAAAACAGAACTCACAAAAGCTTGGACTGGCAAGAACAAACA 69 42	BglII   70 ACAACAAGATCAAAGAAGAAGATCAAATGGCTCTCGAATCACTCCAGTGACCTTGCAA 138 METAlaLeuArgIleThrProValThrLeuGln	NcoI   139 TCGGAGGAGATATCGTTGGTTCTAAGAAGGCTAAATCTCAGATCTCCAAATTGCCATGGCC 207 SerGluArgTyrArgSerPheSerPheProLysAlaAsnLeuArgSerProLysPheAlaMETAla 149 185 201	HindII   208 TCCACCTCGGATCATCCACACCGAAGGTTGACAAATGCCAAGAGCCTTTCAACCTCCACGAGAGGTT 276 SerThrLeuGlySerSerThrProLysValAspAsnAlaLysLysProPheGlnProProArgGluVal 238	BgI   277 CATGTTAGGTGACGCCACTCCATGCCACACAGAAGATAGAGATTTCAAATCCATCGAGGGTTGGGCT 345 HisValGlnValThrHisSerMETProProGlnLysIleGluIlePheLysSerIleGluGlyTrpAla	EcoRV   346 GAGCAGAACATATTGGTTCACCTAAAGCCAGTGGAGAAATGTTGGCAAGCACAGGATTCTTGCCGGAC 414 GluGlnAsnIleLeuValHisLeuLysProValGluLysCysTrpGlnAlaGlnAspPheLeuProAsp
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FIGURE 2  
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415 CCTGCATCTGAAGGATTGATGAAACAAGTCAAGGAACTAAGGGCAAGAGGAAAGCTGATGAT 483  
 ProAlaSerGluGlyPheAspGluGlnValLeuArgAlaArgAlaLysGluIleProAspAsp

484 TACTTGTGTTGGAGATATGATTACAGAGGAAGGCCAACCTTACCTTACCAAAACAATGCTTAAT 552  
 TyrPheValValLeuValGlyAspMetIleThrGluGluAlaLeuProThrTyrGlnThrMetLeuAsn

553 ACCCTAGATGGTGTACGGTGTAGAGACTGGGCTAGCCTTACGCCTGGCTGGACTAGGGCTTGG 621  
 ThrLeuAspGlyValArgAspGluThrGlyAlaSerLeuThrProTrpAlaValTrpThrArgAlaTrp

622 ACAGCTGAAGAACAGGCATGGCGATCTTCTCCACACCTATCTACCTTACCTTCTGGCGGGTAGACATG 690  
 ThrAlaGluGluAsnArgHisGlyAspLeuLeuHisThrTyrLeuTyrLeuSerGlyArgValAspMet  
 626 684

691 AGGCAGATAACAGAACAAATTCACTTACGGTACGGAAATGGATCCTCGTACCGAAAACAGCCCC 759  
 ArgGlnIleGlnLysThrIleGlnTyrLeuIleGlySerGlyMetAspProArgThrGluAsnSerPro  
 736 736

760 TACCTTGGTTCATCACATCGTTCAAGAGCGGCCACATTGTTCTCACGGAAACACGGCCAGG 828  
 TyrLeuGlyPheIleTyrThrSerPheGlnGluArgAlaThrPheValSerHisGlyAsnThrAlaArg

FIGURE 2  
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829 CATGCAAAGGATCATGGGACGTGAAACTGGCCTAAATTGTGGTACAATCGCGCTGACGAAAGCGT 897  
 HisAlaLysAspHisGlyAspValLysLeuAlaGlnIleCysGlyThrIleAlaSerAspGluLysArg  
 833

898 CACGAGACCGCTTATACAAAGATAAGTCGAAAGCTATT CGAGATCGATCCTGGATCCGACCCGTTCTTGCT  
 His Glu Thr Ala Tyr Thr Lys Sile Val Glu Lys Asp Pro Asp Gly Thr Val Leu Ala  
 942

967 TTTGCCGACATGAGGAAAAAGATCTCGATGCCCGCACACTGTACGATGGCGTGTGACAAAC 1035  
PheAlaAspMetMetArgLysSlysIleSerMetProAlaHisLeuMetTyrAspGlyArgAspAspAsn  
BglII 990

036 CTCTCGAACATTTCTGGCGGTGCCAAAGACTCGGGCTACACCGCCAAAGACTACGCCAACATA 1104  
 LeuPheGluHisPheSerAlaValAlaGlnArgLeuGlyValTyrThrAlaLysAspTyrAlaAspIle  
 1077

105 CTGGAAATTCTGGTCGGGGTGGAAAGTGGCGATTGACCGGCCTATCTGGTGAAGGGCGTAAAGCG 1173  
LeuGluPheLeuValGlyArgTrpLysValAlaAspLeuThrGlyLeuSerGlyGluGlyArgLysAla

FIGURE 2  
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	SacI	
1174	CAAGATTATGTTGCCACCAAGAATCAGAAGGCTGGAGGAGAGGCTCAAGGGCGAGCAAAG 1242 GlnAspTyrValCysGlyLeuProProArgLeuGluGluArgAlaGlnGlyArgAlaLys 1228	
	PvuII	
1243	GAAGGACCTGTTGTTCCATTCAAGCTGGATTTCGATAGACAGGTGAAGCTGTGAAGAAAAAACGA 1311 GluGlyProValValProPheSerTrpIleAspArgGlnValIysLeu 1266	
	PvuII	
1312	GCAGTGAAGTTCGGTTCTGTGGCTTATTGGGTAGAGGTAAAAACCTATTAGATGTCCTGCTGT 1380	
1381	AATGTGGTTTTCTAATCTTGAATCTGGTATTGTCGTTGAGTTGGCTGTGTGTAAACTTG 1449	
1450	TGTGGCTGTGGACATATTATAGAACTCGTATGCCAATTGATGACGGTGGTTATCGTCTCCCCCTGGT 1518	
1519	GTTCCTTATTGTT 1533	

FIGURE 2  
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1 AAAAGAAAAAGGTAAGAAAAACAAATGGCTCAAGCTCAATCCTTCTCAAACCCAAAAGT 69  
METAlaLeuLysLeuAsnProPheLeuSerGlnThrGlnLysL

BgIII

70 TACCTCTTCGCTCTCCACCAATGCCAGTACCTAACGATCTCCTAACGATCTACATGCCCTACCCCTCA 138  
euProSerPheAlaLeuProProMETAlaSerThrArgSerProLysPheTerMETAlaSerThrLeuL

139 AGTCTGGTCTAAGGAAGTTGAGAATCTCAAAGAAGCCTTICATGCCCTCCGGAGGTACATGTTCAAGG 207  
ysSerGlySerLysGluValGluAsnLeuLysProProArgGluValHisValGlnV

208 TTACCCATTCTATTGCCA 225  
alThrHisSerIleAla

FIGURE 3A

AAAAGAAAAA	GGTAAGAAAA	AAAACA	ATG	GCT	CTC	AAG	CTC	AAT	CCT	TTC	CTT	TCT	56							
Gln	Thr	Gln	Lys	Leu	Pro	Ser	Phe	Ala	Leu	Pro	Pro	MET	MET	Ala	Leu	Asn	Pro	Phe	Leu	Ser
CCT	AAG	TTC	TAC	ATG	GCC	TCT	ACC	CTC	AAG	TCT	GGT	TCT	110							
Pro	Lys	Phe	Tyr	MET	Ala	Ser	Thr	Leu	Lys	Ser	Gly	Ser	164							
CTC	AAG	AAG	CCT	TTC	ATG	CCT	CGG	GAG	GTA	CAT	GTT	CAG	218							
Leu	Lys	Pro	Pro	Phe	MET	Pro	Pro	Arg	Glu	Val	His	Val	218							
ATG	CCA	CCC	CAA	AAG	ATT	GAG	ATC	TTT	AAA	TCC	CTA	GAC	272							
MET	Pro	Pro	Gln	Lys	Ile	Glu	Ile	Phe	Lys	Ser	Leu	Asp	Asn	Asn	Trp	Ala	Glu	Glu		
AAC	ATT	CTG	GTT	CAT	CTG	AAG	CCA	GTT	GAG	AAA	TGT	TGG	GCT	326						
Asn	Ile	Leu	Val	His	Leu	Lys	Pro	Val	Glu	Lys	Cys	Trp	CAG	CAG	CAG	GAT	TTT			
TTG	CCA	GAT	CCC	GCC	TCT	GAT	GGA	TTT	GAT	GAG	CAA	TGT	380							
Leu	Pro	Asp	Pro	Ala	Ser	Asp	Gly	Phe	Asp	Glu	Gln	Val	Pro	380						
AGA	GCA	AAG	GAG	ATT	CCT	GAT	TAT	TTT	GAT	GAG	GAA	GAA	434							
Arg	Ala	Lys	Glu	Ile	Pro	Asp	Asp	Tyr	Phe	Val	Val	Leu	Asp	Asp	MET	ATA				
ACG	GAA	GAA	GCC	CTT	CCC	ACT	TAT	CAA	ACA	ATG	CTG	AAT	ACC	488						
Thr	Glu	Glu	Ala	Ile	Ile	Pro	Thr	Tyr	Gln	Thr	MET	Leu	Asn	Thr	Leu	Asp	Gly	Val		

CGG GAT GAA ACA GGT GCA AGT CCT ACT TCT TGG GCA ATT TGG ACA AGG GCA TGG	542
Arg Asp Glu Thr Gly Ala Ser Pro Thr Ser Trp Ala Ile Trp Thr Arg Ala Trp	
ACT GCG GAA GAG AAT AGA CAT GGT GAC CTC CTC AAT AAG TAT CTC TAC CTA TCT	596
Thr Ala Glu Glu Asn Arg His Gly Asp Leu Leu Asn Lys Tyr Leu Tyr Leu Ser	
GGA CGA GTG GAC ATG AGG CAA ATT GAG AAG ACA ATT CAA TAT TTG ATT GGT TCA	650
Gly Arg Val Asp MET Arg Gln Ile Glu Lys Thr Ile Gln Tyr Leu Ile Gly Ser	
GGA ATG GAT CCA CGG ACA GAA AAC AGT CCA TAC CTT GGG TTC ATC TAT ACA TCA	704
Gly MET Asp Pro Arg Thr Glu Asn Ser Pro Tyr Leu Gly Phe Ile Tyr Thr Ser	
TTC CAG GAA AGG GCA ACC TTC ATT TCT CAT GGG AAC ACT GCC CGA CAA GCC AAA	758
Phe Gln Glu Arg Ala Thr Phe Ile Ser His Gly Asn Thr Ala Arg Gln Ala Lys	
GAG CAT GGA GAC ATA AAG TTG GCT CAA ATA TGT GGT ACA ATT GCT GCA GAT GAG	812
Glu His Gly Asp Ile Lys Leu Ala Tyr Thr Ile Gln Ile Cys Gly Thr Ile Ala Asp Glu	
AAG CGC CAT GAG ACA GCC TAC ACA AAG ATA GTG GAA AAA CTC TTT GAG ATT GAT	866
Lys Arg His Glu Thr Ala Tyr Thr Lys Ile Val Glu Lys Leu Phe Glu Ile Asp Glu	
CCT GAT GGA ACT GTT TTG GCT GAT ATG ATG AGA AAG AAA ATT TCT ATG	920
Pro Asp Gly Thr Val Leu Ala Phe Ala Asp MET MET Arg Lys Lys Ile Ser MET	
CCT GCA CAC TTG ATG TAT GAT GGC CGA GAT GAT AAT CTT GAC CAC TTT TCA	974
Pro Ala His Leu MET Tyr Asp Gly Arg Asp Asp Asn Leu Phe Asp His Phe Ser	

GCT	GTT	GCG	CAG	CGT	CTT	GGA	GTC	TAC	ACA	GCA	AAG	GAT	TAT	GCA	GAT	ATA	TTG	1028	
Ala	Val	Ala	Gln	Arg	Leu	Gly	Val	Thr	Ala	Lys	Asp	Tyr	Ala	Asp	Ile	Leu			
GAG	TTC	TTG	GTG	GGC	AGA	TGG	AAG	GTG	GAT	AAA	CTA	ACG	GGC	CTT	TCA	GCT	GAG	1082	
Glu	Phe	Leu	Val	Gly	Arg	Trp	Lys	Val	Asp	Lys	Leu	Thr	Gly	Leu	Ser	Ala	Glu		
GGA	CAA	AAG	GCT	CAG	GAC	TAT	GTT	TGG	TTA	CCT	CCA	AGA	ATT	AGA	AGG	CTG	1136		
Gly	Gln	Lys	Ala	Gln	Asp	Tyr	Val	Cys	Arg	Leu	Pro	Pro	Arg	Ile	Arg	Arg	Leu		
GAA	GAG	AGA	GCT	CAA	GGA	AGG	GCA	AAG	GAA	GCA	CCC	ACC	ATG	CCT	TTC	AGC	TGG	1190	
Glu	Glu	Glu	Glu	Ala	Gln	Gly	Arg	Ala	Lys	Glu	Ala	Pro	Thr	MET	Pro	Phe	Ser	Trp	
ATT	TTC	GAT	AGG	CAA	GTG	AAG	CTG	TAGG	GGCTA	AAGT	GGAGA	CGAA	ACCGAA	ATGG	TTAGTT		1254		
Ile	Phe	Asp	Arg	Gln	Val	Lys	Leu												
TCAC	CTCT	TTT	TCAT	GCCC	CAT	CCCT	GCAGAA	TCAGAA	AGTAGT	AGGTAGA	ATT	TGTTAGTT	GC	TTTTTT	TTA		1324		
CAAG	TG	CCAGT	TTAG	TTAAG	GTCT	GTG	GGAA	GGGAGT	AGT	TGAGGAGT	GA	ATTAGTA	AG	TTGTAGATA	AC		1394		
AGT	TG	TTT	TCT	TGTG	TGT	TCA	TGAGT	TATG	GCT	GATAGAG	AGC	TGAGT	GTG	TGTTGTTG	T		1464		
ATGG	TCT	CTT	TCT	CTT	GTG	AGTT	CTT	TTT	CTT	CTT	CTT	CTT	CTT	CTCT	CTCT	CTCT	1534		
CTCT	TTT	TCT	TCT	CTT	CTT	CTT	CTT	CTT	CTT	CTT	CTT	CTT	CTT	CTT	CTT	CTT			
TGAT	CAG	TCT	ATCCC	AA	GTG	TCT	CAAG	TATA	AGC	AAAC	GAT	CCA	TGTG	GAATT	TTGAT	ATGG	1604		
CAC	AACT	TGA	TCT	TTTG	TCT	TCT	TCT	TCT	TCT	TCT	TCT	TCT	TCT	TCT	TCT	TCT			
TGAA	AAA	AA	ACAC	GGC	CTG	TTG	TTG	TTG	TTG	TTG	TTG	TTG	TTG	TTG	TTG	TTG	1668		

PCGN3235

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1 TGAGAGATAAGTGTGAGAGCATTAGCCTTAGAGAGAGAGAGCTTGTGTCTGAAAGAACAA 69  
HindIII  
|  
70 ATGGCATTGAAGCTAACCCCTTGGCATCTCAGCCTTACAACTTCCCT 117  
METAlaLeuLysLeuAsnProLeuAlaSerGlnProTyRAsnPhePro

FIGURE 4A

**PCGN3236**

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Pst I

1 ACTCATGGCTATTGGACAAGAGCTTGGACTGAGAGAACCGACACGGTGAATCTCAATAAG 69  
ThrSerTrpAlaLeTrpThrArgAlaTrpThrAlaGluGluAsnArgHisGlyAspLeuAsnLys

70 TATCTTACTTGTCTGGACGTGTTGACATGAGGCAGATTGAAAGACCATTCACTTGATTGGTCT 138  
TyrLeuTyrlLeuSerGlyArgValAspMETargGlnIleGluLysThrIleGlySer

BamHI

139 GGAATGGATCCTAGAACAGAGAACAAATCCTTACCTCGG 176  
GLYMETAspProArgThrGluAsnAsnProTyrlLeuAla

FIGURE 4B

**PCGN3235**

TGAGAGATAG TGTGAGAGCA TTAGCCCTAG AGAGAGAGAG AGAGAGCTTG TGTCTGAAAG AATCCACAA

ATG GCA TTG AAG CTT AAC CCT TTG GCA TCT CAG CCT TAC AAC TTC CCT TCC TCG  
 MET Ala Leu Lys Leu Asn Pro Leu Ala Ser Gln Pro Tyr Asn Phe Pro Ser Ser

GCT CGT CCG CCA ATC TCT ACT TTC AGA TCT CCC AAG TTC CTC TGC CTC GCT TCT  
 Ala Arg Pro Pro Ile Ser Thr Phe Arg Ser Pro Lys Phe Leu Cys Leu Ala Ser

TCT TCT CCC GCT CTC AGC TCC AAG GAG GTT GAG AGT TTG AAG AAG CCA TTC ACA  
 Ser Ser Pro Ala Leu Ser Ser Lys Glu Val Glu Ser Leu Lys Lys Pro Phe Thr

CCA CCT AAG GAA GTG CAC GTT CAA GTC CTG CAT TCC ATG CCA CCC CAG AAG ATC  
 Pro Pro Lys Glu Val Val Gln Val Leu His Ser MET Pro Pro Gln Lys Ile

GAG ATC TTC AAA TCC ATG GAA GAC TGG GCC GAG CAG AAC CTT CTA ACT CAG CTC  
 Glu Ile Phe Lys Ser MET Glu Asp Trp Ala Glu Gln Asn Leu Leu Thr Gln Leu

AAA GAC GTG GAG AAG TCG TGG CAG CCC CAG GAC TTC TTA CCC GAC CCT GCA TCC  
 Lys Asp Val Glu Lys Ser Trp Gln Pro Gln Asp Phe Leu Pro Asp Pro Ala Ser

GAT GGG TTC GAA GAT CAG GTT AGA GAG CTA AGA GAG AGG GCA AGA GAG CTC CCT  
 Asp Gly Phe Glu Asp Gln Val Arg Glu Leu Arg Glu Arg Ala Arg Glu Leu Pro

GAT GAT TAC TTC GTT GTT CTG GTG GGA GAC ATG ATC ACG GAA GAG GCG CRT CCG  
 Asp Asp Tyr Phe Val Val Leu Val Gly Asp MET Ile Thr Glu Glu Ala Leu Pro

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FIGURE 4C  
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ACC	TAT	CAA	ACC	ATG	TTC	AAC	ACT	TTG	GAT	GGA	GTG	AGG	GAT	GAA	ACT	GGC	GCT
Thr	Tyr	Gln	Thr	MET	Leu	Asn	Thr	Leu	Asp	Gly	Val	Arg	Asp	Glu	Thr	Gly	Ala
AGC	CCC	ACT	TCA	TGG	GCT	ATT	TGG	ACA	AGA	GCT	TGG	ACT	GCA	GAA	GAG	AAC	CGA
Ser	Pro	Thr	Ser	Trp	Ala	Ile	Trp	Thr	Arg	Ala	Trp	Thr	Ala	Glu	Glu	Asn	Arg
CAC	GGT	GAT	CTT	CTC	AAT	AAG	TAT	CTT	TAC	TTG	TCT	GGA	CGT	GTT	GAC	ATG	AGG
His	Gly	Asp	Leu	Leu	Asn	Lys	Tyr	Leu	Tyr	Leu	Ser	Gly	Arg	Val	Asp	MET	Arg
CAG	ATT	GAA	AAG	ACC	ATT	CAG	TAC	TTG	ATT	GGT	TCT	GGA	ATG	GAT	CCT	AGA	ACA
Gln	Ile	Glu	Lys	Thr	Ile	Gln	Tyr	Leu	Ile	Gly	Ser	Gly	MET	Asp	Pro	Arg	Thr
GAG	AAC	AAT	CCT	TAC	CTC	GGC	TTC	ATC	TAC	ACT	TCA	TTC	CAA	GAA	AGA	GCC	ACC
Glu	Asn	Asn	Pro	Tyr	Leu	Gly	Phe	Ile	Tyr	Thr	Ser	Phe	Gln	Glu	Arg	Ala	Thr
TTC	ATC	TCT	CAC	GGA	AAC	ACA	GCT	CGC	CAA	GCC	AAA	GAG	CAC	GGA	GAC	CTC	AAG
Phe	Ile	Ser	His	Gly	Asn	Thr	Ala	Arg	Gln	Ala	Lys	Glu	His	Gly	Asp	Leu	Lys
CTA	GCC	CAA	ATC	TGC	GGC	ACA	ATA	GCT	GCA	GAC	GAG	AAG	CGT	CAT	GAG	ACA	GCT
Leu	Ala	Gln	Ile	Cys	Gly	Thr	Ile	Ala	Ala	Asp	Glu	Lys	Arg	His	Glu	Thr	Ala
TAC	ACC	AAG	ATA	GTT	GAG	AAG	CTC	TTT	GAG	ATT	GAT	CCT	GAT	GGT	ACT	GTG	ATG
Tyr	Thr	Lys	Ile	Val	Glu	Lys	Leu	Phe	Glu	Ile	Asp	Pro	Asp	Gly	Thr	Val	MET
GCG	TTT	GCA	GAC	ATG	ATG	AGG	AAG	AAA	ATC	TCG	ATG	CCT	GCT	CAC	TTG	ATG	TAC
Ala	Phe	Ala	Asp	MET	MET	Arg	Lys	Lys	Ile	Ser	MET	Pro	Ala	His	Leu	MET	Tyr

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GAT GGG CGG GAT GAA AGC CTC TTT GAC AAC TTC TCT TCT GTT GCT CAG AGG CTC  
 ASP G1Y Arg ASP Glu Ser Leu Phe ASP Asn Phe Ser Ser Val Ala Gln Arg Leu  
  
 GGT GTT TAC ACT GCC AAA GAC TAT GCG GAC ATT CTT GAG TTT TTG GTT GGG AGG  
 G1Y Val Tyr Thr Ala Lys ASP Tyr Ala Asp Ile Leu Glu Phe Leu Val Gly Arg  
  
 TGG AAG ATT GAG AGC TTG ACC GGG CTT TCA GGT GAA GGA AAC AAA GCG CAA GAG  
 Trp Lys Ile Glu Ser Leu Thr G1Y Leu Ser G1Y Glu G1Y Asn Lys Ala Gln Glu  
  
 TAC TTG TGT GGG TTG ACT CCA AGA ATC AGG AGG TTG GAT GAG AGA GCT CAA GCA  
 Tyr Leu Cys G1Y Leu Thr Pro Arg Ile Arg Arg Leu Asp Glu Arg Ala Gln Ala  
  
 AGA GCC AAG AAA GGA CCC AAG GTT CCT TTC AGC TGG ATA CAT GAC AGA GAA GTG  
 Arg Ala Lys Lys Gly Pro Lys Val Pro Phe Ser Trp Ile His Asp Arg Glu Val  
  
 CAG CTC TAA AAAGGAA CAAAGCTATG AAACCTTTTC ACTCTCCGTC GTCCCTCAT TGATCTATCT  
 Gln Leu \*

GCTCTTGAAA TTGGTGTAGA TTACTATGGT TTGTGATATT GTTCGTGGGT CTAGTTACAA AGTTGAGAAG  
 CAGTGATTAA GTAGCTTTGT TGTTTCCAGT CTTAAATGT TTTTGTTT GGTCCCTTTA GTAAACTGT  
 TGTAGTTAAA TCAGTTAAC TGTTGGTCT GT

FIGURE 4C  
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GAT	GCC	AAA	ANG	CCT	CAC	ATG	CCT	CCT	AGA	GAA	GCT	CAT	GTG	CAA	AAG	48
Asp	Ala	Lys	Xaa	Pro	His	MET	Pro	Pro	Arg	Glu	Ala	His	Val	Gln	Lys	
1						5										15
ACC	CAT	TCA	ATK	CCG	CCT	CAA	AAG	ATT	GAG	ATT	TTC	AAA	TCC	TTG	GAG	96
Thr	His	Ser	Xaa	Pro	Pro	Gln	Lys	Ile	Glu	Ile	Phe	Lys	Ser	Leu	Glu	
								20					30			
GGT	TGG	GCT	GAG	GAG	AAT	GTC	RTG	GTG	CAT	CTT	AAA	CCT	GTG	GAG	AA	143
Gly	Trp	Ala	Glu	Glu	Asn	Val	Leu	Val	His	Leu	Lys	Pro	Val	Glu		
									35			40				45

FIGURE 5

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**Amino Acid Sequence From Fragment F2**

K	E	I	P	D	D	Y	FVVLVGD <sup>MITEE</sup> ALP <sup>TY</sup>	Q	T	M	L	N	T
AAA	GAA	AUU	CCN	GAU	GAU	UAU	CAA	ACN	AUG	CUN	AAU	AC/N	
G	G	C	C	C	C	C	G						

A

## Forward Primers:

5' GCTTAAGCTT AAP GAP ATQ CCA GAQ GAQ TA3' Desat 13-1  
 A CCG Desat 13-2  
 CCC Desat 13-3  
 CCT Desat 13-4

Reverse Primers:  
(complements)

Desat 13-5a 3' GTQ TGN TAC GAN TTP TGCTTAAGCGA 5'  
 Desat 13-6a AAQ

Oligonucleotides

P = A or G

Q = T or C

N = A, C, T or C

FIGURE 6

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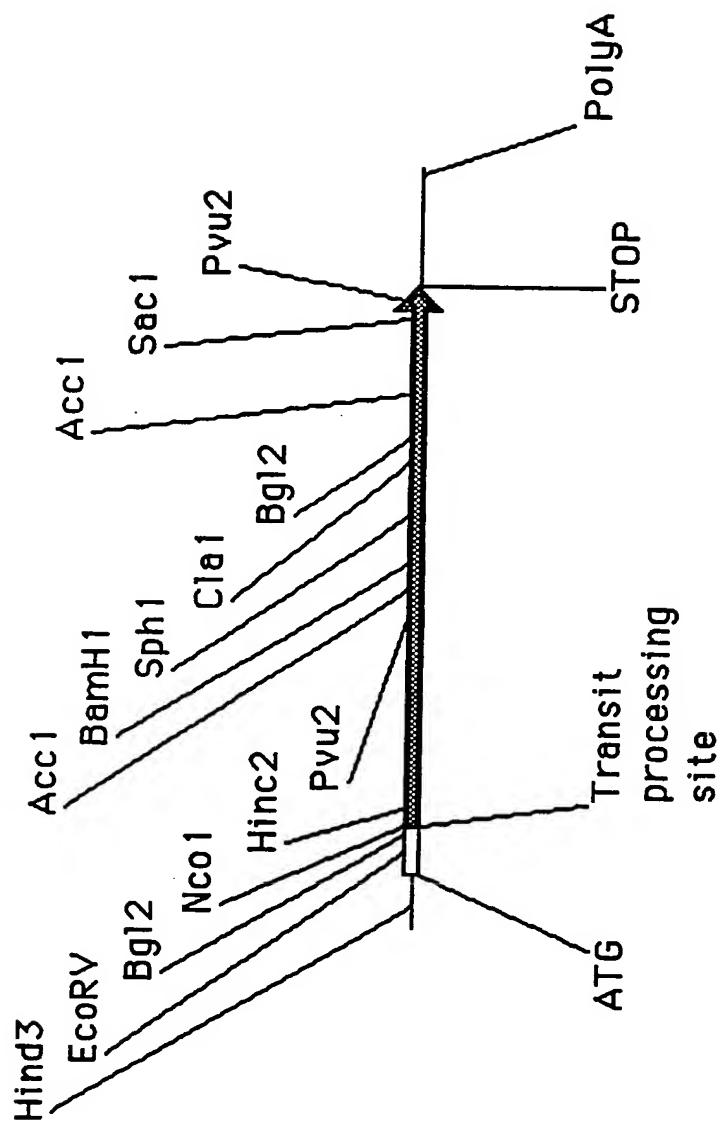


FIGURE 7A

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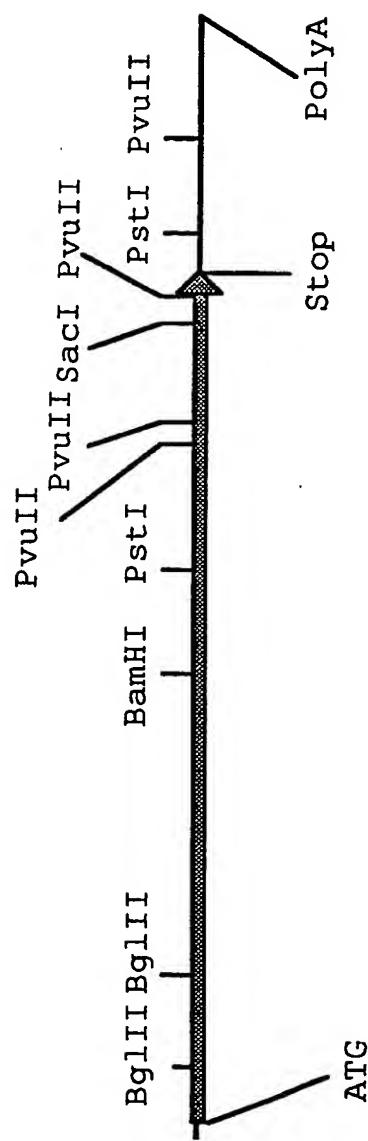


FIGURE 7B

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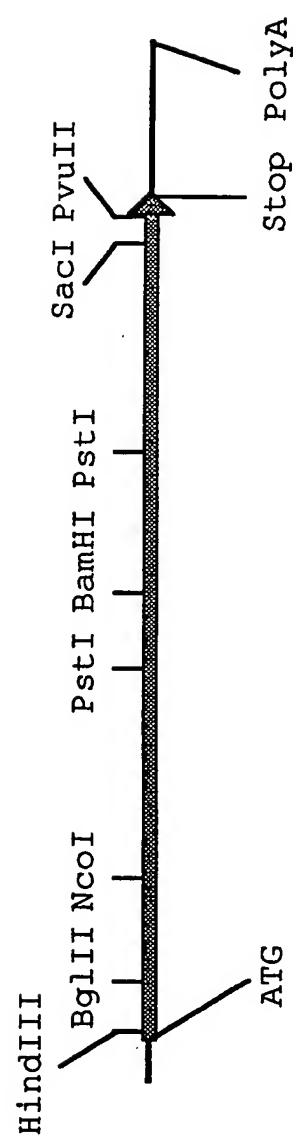


FIGURE 7C

TCTAGAATTCTCTAATTACG	TCTGTTTGTTCATTTTTA	TATGATATCA	AATATCGTC	ATAAATATAT	70	
GGTTTAGATGCCAAAAAAT	TATTTACTTG	GTGAATAAAT	TACGTTAAAT	ATTAGAAATA	CATCATTAG	140
TTAAATAATACCAAAAC	CAAAATTCATCA	TATCCGGCT	GGCGCGGGT	CAGGGTCTCG	TTAGTTTAA	210
AATCAATGCA	GTTCACAAATT	ATTTCACCGC	TGAAATAAG	TATAATTGT	ATTGAAATA	280
TTTTTGTGT	AACAAATATT	TGTGTACAC	AGAATTAA	AAAAAACAG	AAAATACTCA	350
TAATAAAAAA	ATTAAATTGA	GTAGAAAT	TGTGTACCA	ATAACAAAG	ATTTATGG	420
TCAACACACC	ATAAACACAA	GACTTTAA	AAATTAAAG	ATAATAAG	CAATAACAAT	490
AATTCTCAA	ATCCTTAA	ATCAATCTCC	CACTTTAAT	CCCCCTTAGT	TTTAGTTGGT	560
TTTGTGACT	ACCGTATTGT	AACTTTGTC	AAATTGTCAT	AAATACTGT	CAAACCTCTGG	630
GTCTGCTACA	TCTGTCTTT	ATTTATAAA	CACAGCTGT	AAICAGAAATT	TGGTTTATTA	700
CTGCACGAAA	CTTGTGTGAG	CATATTGT	CTGTTCTGG	TTCATGACCT	TCTTCCGCAT	770
TGTAATGGCC	ACTTGCAAGA	GGGTTCTTC	AACGAGATAA	GTGGAACAAA	TATTGTCCG	840
ATATAAAATC	TCCCCATCTC	TATATAAAAT	ACCAAGCATTC	ACCATCATGA	ATACCTCAAA	910
ACAAATACTT	CAATAAAAG	ACCAAAAAAA	ATAAAGCAA	AGAAAAGCCT	TCTTGTGCAC	980

GAAGCCCTCT AGGTTTCAC GAC ATG AAG TTC ACT ACT CTA ATG GTC ATC ACA TTG 1036  
 MET Lys Phe Thr Thr Leu MET Val Ile Thr Leu  
 GTG ATA ATC GCC ATC TCG TCT CCT GTT CCA ATT AGA GCA ACC ACG GTT GAA AGT 1090  
 Val Ile Ala Ile Ser Ser Pro Val Pro Ile Arg Ala Thr Thr Val Glu Ser  
 TTC GGA GAA GTG GCA CAA TCG TGT GTG ACA GAA CTC GCC CCA TGC TTA CCA 1144  
 Phe Gly Glu Val Ala Gln Ser Cys Val Val Thr Glu Leu Ala Pro Cys Leu Pro  
 GCA ATG ACC ACG GCA GGA GAC CCG ACT ACA GAA TGC TGC GAC AAA CTG GTA GAG 1198  
 Ala MET Thr Ala Gly Asp Pro Thr Thr Glu Cys Cys Asp Lys Leu Val Glu  
 CAG AAA CCA TGT CTT TGT GGT TAT ATT CGA AAC CCA GCC TAT AGT ATG TAT GTT 1252  
 Gln Lys Pro Cys Leu Cys Gly Tyr Ile Arg Asn Pro Ala Tyr Ser MET Tyr Val  
 ACT TCT CCA AAC GGT CGC AAA GTC TTA GAT TTT TGT AAG GTT CCT TTT CCT AGT 1306  
 Thr Ser Pro Asn Gly Arg Lys Val Leu Asp Phe Cys Lys Val Pro Phe Pro Ser  
 TGT TAAATCTCTC AAGACATTGC TAAGAAAAAT ATTATTAAAATAAAAGAAT CAAACTAGAT 1369  
 Cys  
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 CTGATGTAAC ATGAAATCAT CATGTTATGG TTGAAAGCTTA TATGCTGAAG TGTTTGATT TATATATGTG 1439  
 TGTGTGTGTG TCCTGCTCAA TTGTTGAAAC ACACACGTTT CTCCTGATT GGATTAAAT TATATTGTA 1509  
 GTTAAAAAAA AGAAAAAGAT GGAATGCTAT TTATACAAGT TGATGAAAAA GTGGAAGTAC AATTAGATA 1579

TCTCCTACAC TTAAAGAATG AAACAATAAT AGACTTACGA AACAAATGAA AAATACTAA ATTGTCGACA 1649  
 ATCAAACGTCC GATGACGAGT TTATTATTA AAATTGTGT GAAGGACTAG CAGTTCAACC AAATGATATT 1719  
 GAACATATACT ATCAACAAAT ATGATAATCA TAAAGAGAG AATGGGGGG GGGTGTGTT TACAGAAAC 1789  
 CTCTTTCT CAGCTCGCTA AAACCTAAC ACTAGAGACC TAGCTCTGAC CGTGGCTCA TCGGTGCCGG 1859  
 AGGTGTAACC TTCTTCTTCCC ATGACCCGAA ACCTCTCTTT CCCAACTCAC GAAAACCCTA CAATCAAAA 1929  
 CCTAGCTCCG ACCGTGGCT CATCGGTGCC GAAGGTGTAA CCTTTCTCTC CCATCATAGT TTCTCGTAAA 1999  
 TGAAAGCTAA TGGGCAATC GATTTTAA TGTTAACC ATGCCAAGCC ATTCTTATA GGACAATTGTT 2069  
 CAATAATAGC ATCTTTGAG TTTGTCTCA AAAGTGACAC TAGAAGAAA AAGTCACAAA AATGACATT 2139  
 ATTAAAAGT AAAATATCCC TAATACCTT GGTTAAATT AAATAAGTAA ACAAAATAA ATAAAACAAA 2209  
 ATAAAATAAA ATAAAAAAAT GAAAAAAAGA ATTTTTTA TAGTTTCAGA TTATATGTT TCAGATTGGA 2279  
 ATTTTTAA ATTCCCTTT TAAATTTC TTGAAATTGAA TTTTTTTTGAAATTGTT TGAAACTGTT 2349  
 TTAAATTTT TTATTTAA TTTTTAGTA TTATTTTT ATTAAATAA ATTAAACG CTAAATTCCAA 2419  
 ACTCCCCCCC CCCCCAATCT CTCCTAGTCT TTCTCTTTT CTTATATTG GGCTTCTATC 2489  
 TTCTCTTTT TTTCAAGGCC CAAAGTATCA TGTGTAACAA CGGGTGTCA AAAACGGGCC CGCCCTGGCCG 2559

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 FIGURE 8  
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TTTACTCGCC CGATTAATG ATGATCGGAA GGCTGCCATG GCGAGGGAA GGTAAATCAGT GGTCTAGGC 2629  
 GCTGAACTA GAAAACCTTC AAAAATCGAA ATTAAAGAG CTAATCGGT GTTATCTCA TGAATCTATT 2699  
 ATATTAGTT GAAACTCACA AGAATCGGT GTAAAAACTA TGAATCGTG CAAAAAAAT GAAGAACAAA 2769  
 ATATTCTCAG ATCTGGAAA CACAGAGAAG AGGTGAAGA TGAGGGTAAA ATCGTATTGTCATT 2839  
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 AAACTAAAT CAAAAAAA TGATGCAAAA TTCAATGATA ATAACTCGAA CTCGCAACCA TATGCATCTT 2909  
 TAGACTGCGA CACGGACCCAC TAGACTAAGC ATTAAATG TTATTCTAC ACAGACCTAA TATAATGTCTA 2979  
 AAACTAGGGCG CCGAGTACGC CCCGCTTAAT CCCGAGTTT TTGTTAGCTCG CTAGACCCAG GGTTCACCGCC 3049  
 CGACTAACGA GTAGGGTAAT TCTGAACCTGG GTTAACACA TAGAGAACAT CGGGGACCCCT TCCCTGCCGA 3119  
 TGATGCGCC TCCGATGAAAC TTCCTGTAAC GCCTTCAGTT TCCATTGATT TTCCCCTTTA ATCTGATCAG 3189  
 TTCCATGTT TATCCAACTC ATCCCACCTCC GTAGCATTTA ATCGATCTCA TCATTACAT ACATAACCAG 3259  
 TAGGAGGTCT CATAAAATT TGAACGTTTC CAGCGATGAA CAGTGCCAAAT CTCTGGGAA TCCATTCTC 3329  
 TAAGCTCAGG GCTGGGGGCT GCAGCCCCGG GATCCCACTAG TTCTAGGGGG CCCCACCGCG GTGGAGCTCC 3399  
 AATTTCGGCCCT ATAGTGAAGTC GTATTACGCG CGCTCACTGG C 3440

Xba I

1 CTCGAGGCTGAAGGATTTTAGAGATTCAACGACAGATGGACCCCTCCACTAGGCCAACTGC 69

2

70 AGAACCTAACAAATGCAAATACACTCCTCAGCCTCAAGGAGCCTTAATAGGACTGGAAACAAGCG 138

Bgl II

1 139 GTCAAGTGAAGTAAATTTCCTTCCAAGATAGATCTATGGTTCGTTCATGAAGTTGTGGTTAAATT 207  
169

208 GTGTAGCAACAGGATAAGTGCAGAAGTGAAGAATAGAGTTCGACCTCATCTACCTACCCGGAAACCTCTGAAT 276

277 GTATCCCCATTGAAAGAAGAGGGCAAATCTGCACCCAGAAGGATAAAGAAATTGGACGCCTGAA 345  
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346 GAAAGCTGAGGTTCTGAGGGAAAGGAGTAAAGAGTATGTCTACTACTACTATAATCAAGTTCAA 414

415 GAAAGCTGAGGCTGGCTCTCACTTTATATGTTGATGTTGTTGCAGGTATGGTAATCATGGAAAGAG 483

484 ATAAAGAATGCAAACCCCTGAAGTATTGGCAGAGGACTGAGGTGAGAGGATGTCACTTTGTGTTA 552

553 CTCATCTGAATTATCTTATATGCGAAATTGTAAGTGGTAACCTTGTAACTTTGGTAGGTGGAT 621

622 TTGAAGGATAATGGAGGAACCTGCTCGGTAGGGTAACAAGTTTATATTGCTATGAAGTTTTTG 690

691 CCTGGCTGACGTATCAGCAGCTGGGAGAGTGGTATTAGAAAGGGTCTTTCACATTGTGTTGTG 759

FIGURE 9  
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760 ACAAAATATAATTGGCCGGTATGGTTGGTTAAGACTTGTGAGAGACGTGGGGTTTTGATGTA 828

829 TAATTAGTCTGTGTTAGAACGAAAGACTTGTGCGTATGCTTTAACCTGAGGGGTTGTT 897

Bg I

898 GTGTTAGGAACTTGACTTGTGACTTGTGACTTGTCTCTCAAGATCTGATTGTAAGGTCTGGTGGTAGTA 966  
937

967 CTGTTGGTTAATTGGTTGACTATTGAGTCACTGGCCCATGACTTTAAATTAGGCTGGTATAT 1035

1036 TTTTTGGTTAAACCGGTCCTGAGATAAGTGCATTGCAATTTCAGTCAATTAAATTCTCAAGGTAAT 1104

1105 GGCCTGAATACTTGATAGTTAACAGACTTAACAGGCCTAAAGGCCATGTTATCATAAACGTCAAT 1173

Hind III

1174 TGTAGACTGGCACCAAGCTTAAATGTAGCCAGGCCCTTAAAGACTTAACAGGCCTAAAGACTT 1242  
1190

1243 AACATTCCCTAAAGGCCCATGTTATCATAAAACGTCACTCGTTTGAGTGCACCAAGCTAAATGTAGGCC 1311

1312 AGGCCTAACAGACTTAACAGGCCTAAAGGCCATGTTATCATAAACGCCGTGCGTTGAGTCAC 1380

FIGURE 9  
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2071 TCACTCAAAGACGACCAAAAGGTCGGGGAGACCAAGTTGGCTGATCTGGAGCCATTCTCGAC 2139  
2140 ACTGTAAGTCATCAATCATCTCTTATGTGAATAAGAGAACTTGAAGAGTTGTTAACATATTAA 2208  
EcoRV  
|  
2209 CTGAGTGTGTTGCATGCAGGTTGAGATAGTGGATGGGTTAGAGGAAGAGTTGATATGGCTGA 2277  
2264  
SstI  
|  
2278 AGAGAAAGCTCAGAAGATTCGCTACTGTGGAGGAAGCTGCTGAACATCATGAAGAGCTCGTCAACTTAA 2346  
2335  
2347 GAAGTAATTAGTATTAAAGAGCAGCCAAAGGCTTGTGGTTGTTCTCATATAATCTTCCTGTCTCAT 2415  
2416 TTTCTTTCTTTAATGTGTCAAGCCGACTCTGTGGTTAAAGTAGTATCTGTTGCCATGGATCTCTC 2484  
HindIII  
|  
2485 TCTATTTGTCGACTGAAACCTTTGGTTACACATGAAGCTTGTCTTCTTAATCGAAAT 2553  
2493 2523  
2554 GCCAAATGGGAGATAGGGAAATCTTGTATTAAACACATACATAAGTCAANGAGTAGGCCCTAAGATGACA 2622  
2623 ATTATAAACAAATCCTATTACACATTGATATACAGGTATGATATTCCAAATCAGCGTCAAAGAAATCC 2691

2692 AGCATCTTCATCTGAATAAGACATTCTCCAAGTCACATCTTCCTGCACCAAAACCAAGTA 2760  
2761 CTAATCATGAAACATTGCAATAATCACATGCCCTAGGGAGAGTTGGTGTAGTGGTGTAGT 2829  
2830 GATTAATGAGGGCTAGAGGGTTAAGAAGGTTAACCTGGAAAGAAAGTCTGCAAGGAAAGTAACTAGA 2898  
2899 GAAGAGGAAAGATAAGGAGTGGTAACAAACACTTGTGATCCCATAACAGCCTCCAGCATTTCAAATGTT 2967  
2968 ATTTCCTTACATAAGAACAAAGAGAAGTCTGACTAGATGATATTATAAGGATAAGTGTGTTACCAT 3036  
3037 AGGCCAAAGTGAAGGCCGTTGCAAGAGCTAACCGAGACAGTACACGTTGGCATATACTCATCAACAT 3105  
3106 GATCTGAAAGTAAACATAACAGTTAACACATAATGAAACACATAATGGTTACCTTGAGAAGCAAATCAAGACCTATA 3174  
3175 ACAAGCCCCAGAGATGAGGAAGTCCGTGTCAACGCTTCACCGCCATTGGCTAGTTCCCTGGAAAGACA 3243 28/42  
3244 AGGGCCACCAACCAACCTAACTTCAAAACTCCAGAAACAAACACTCCAAATGTTGTCAACAAAGTCATAAGATTCGA 3312  
3313 AACTACTTGTACAGGGTTGTATAGATAATATAAGAATAATAGTGGAAAGATAGTATAAAATAAAATAAA 3381  
3382 TAAAGATTCCTATCGGTAAATAGTTATAATATCGGGGCTATATAAGTATAAAAGAAACTCTTC 3450  
3451 CAATCCGACCGTTGAAATCACTCTCAATCTGGCTAACGACCGGATCGTTGCCGTAAATTTTCGC 3519  
3520 TGCTATAATAAGAACCTTCCTCTGTTCTCGATCAAATTTTTGGAAAAATTAAAGTTTGAA 3588

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3589 TCTATCGTAGATGCTGTGACAAAAAAATTGTTTATCGAAAGATGAGAAACATGAGGCCCTGTTCATGC 3657  
BamHI  
|  
3658 AGGAACAGACCACGGATCCATCTCGCCGATGACGCTCCTCTGATGAAATCGTCACGCACGGAC 3726  
3674

BamHI  
|  
3727 AGGATCCAACCGCTGGACCACGGCATCTAACGCCAAGAACGACGAAAGCAGCAGCTCAGAGACTCGC 3795  
3729

3796 GGCTGTGATGTCGAACCAAACAGGGGACCGATGAAGACAGTGATGACCTTCTTGAATACAAACGC 3864

BglII  
|  
3865 TGTCGGAAGGCATTGGTCTCGCTGCCGAAGATCT 3898  
3894

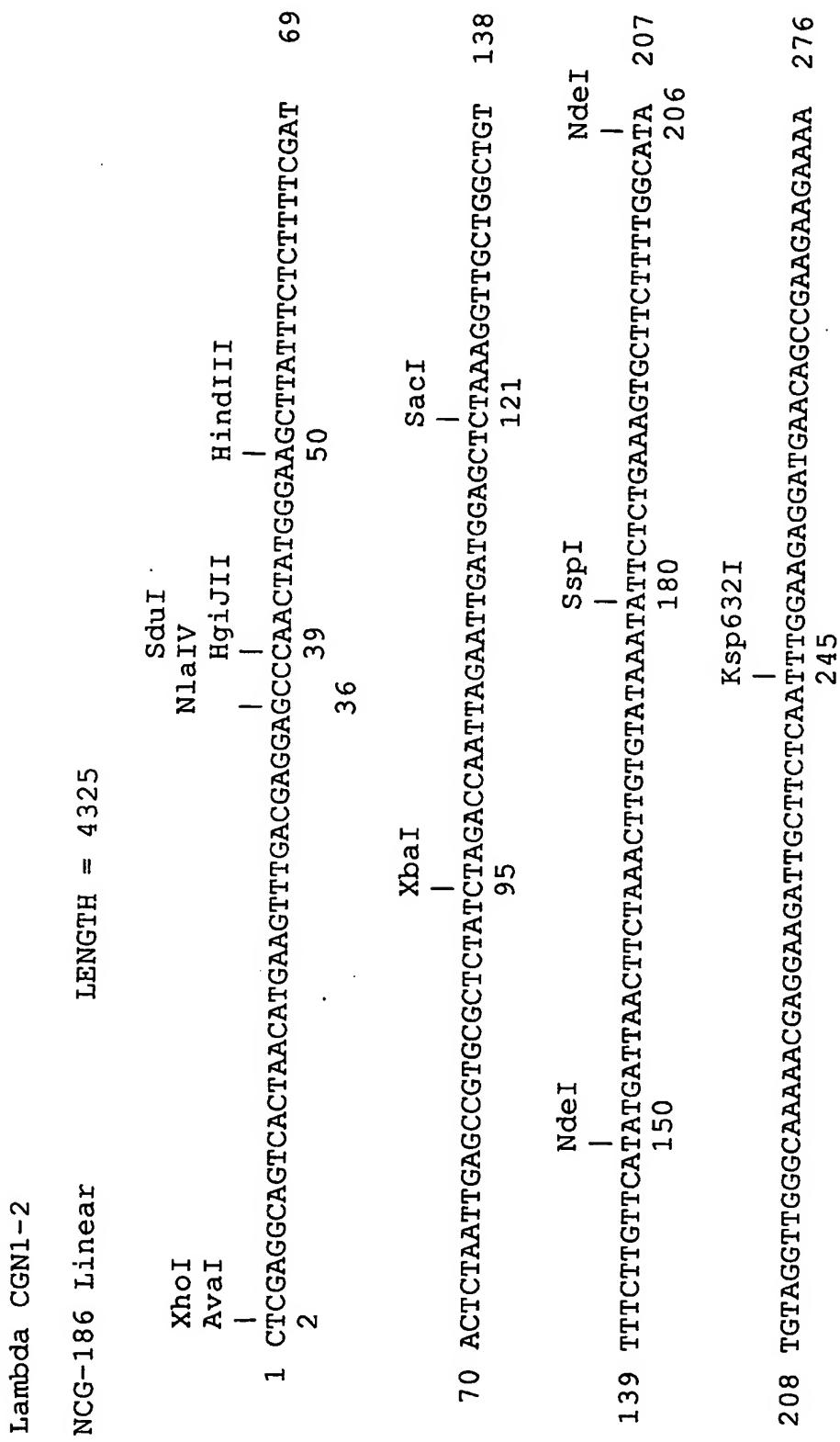


FIGURE 10  
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277 TAAGAATAGGCAGTCCTGCTACTCAAATGGATCTCAGTCTATAACGGTCGTCCATGAAACAGAGGT  
 305  
 XbaII

346 AAAACATTGGCATATACACTTGAAGTTCCTCACTAACTGTGAAATCTTTGGTAGATATCACTA 414  
 MmeI | EcoRV |  
 | |  
 401 408

415 CAATGTCGGAGACAA3GGCTGMNCANCATATACAAAAGGGAAATGAAAGATGGCTTTGATTAGCTG 483 31/42  
 SduI | HaeI |  
 MstI | |  
 BclI HgiAI |  
 | |  
 437 442 469  
 439

484 TGTAGCATAGCAGCTAATCTCTGGGCTCATGCTGGAAACTGGATTCACTTCAAGTTA 552  
 SduI | HgiJII |  
 | |

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Cfr10I  
 BbvII  
 |  
 553 TGAGTTGTACCGGTCTTACACAAAGGTAATAATCAGTTGAAGCAATTAAAGAATCAATTGATTGTTG 621  
 560 563

622 AGTAAGAAACTTACCTTATGTTTCCCCGCAGGACTGGATTATGGAACAATGGAAAGAAC 690

SacI  
 |  
 691 TACTATATAAGCTCCATAGCTGGTTCAAGATAACGGGAGCTTTAGTTGTTATGTCAAAAGGTAGTGT 759  
 731

BbvII  
 |  
 760 TTAGTGAATAAAACTTACCAAAAGTCTTCATTGACTTTATTATAACTTGTGAAATGCTAG 828  
 782

829 GAACTACTTATTCTCAGCAGTCATAAAAGTGACTCATTTCCGTTCAAGTGGATAATAAGAAAT 897

898 GGAAAGAAGATTTCATGTAACCTCCATGACAACACTGCTGGTAATCGTGGGTGTGGTAATGTCGAGGA 966

BclI  
 |  
 967 ACTCTGGCTTCTGATCAGGTAGGTTTGTCTCTTATTGTTGTTTATTTCCTGATAGT 1035  
 981

FIGURE 10  
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1036	CTAATATGATAAACTCTGGCTTGTGAAAGGTGGAGCTTGACTTTGTACCCCAAGGGATGGGATAAC	1104
1105	ATAGGAGGTGGAGAATGGGTATAGAATAACATCAAATGGCAGCAACTGCGGATCAAGCAGCTTCAATAT	1173
	Tth111II	
1174	TAAGCATAACCAAAAGCGTAAGATGGTGGATGAAACTCAAGAGACTCTCCGCACCCGCCCTTCCAAGTA	1242
1175		1242
	XbaII	
1243	CTCATGTCAAGGGTTGGTTCTTAGCTTGAACACAGATTGGATCTTTGTTTCCATATACT	1311
	1285	
1312	TAGGACCTGAGGAGCTTGTGATTTCAGGACAAATGGCGAAGAATCTGTACATTGCATCA	1380
	Af1II	
1381	ATATGCTATGGCAGGACAGTGTGCTGATAACACACTTAAGCATCATGGAAAGACAATTGGAG	1449
	1415	
1450	CGAGACTCAGGGTCGTCTATAATACCAATCAAAGACGTAACCAACCTCTTGGTTGAATGTA	1518
	SspI	
1519	ATGAAAGGGATGTGTCTGGTATGTACGAATAACAAAGAGAAGATGGAATTAGTAGTAAATA	1587
	1587	

FIGURE 10  
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1588 TTTGGGAGCTTTAAGCCCTCAAGTGTGCTTTATCTTATTGATATCATCCATTGCGTTGTTAA 1656  EcoRV   	1635  Xba I   	1657 TGGTCTAGATATGTCCTATATCTTCAGTGTCTGATAAGTGAGAAAACCATACCAA 1725    	1664  Ssp I   	1726 ACCAAATATTCAAATCTTAAATAATGTTGAATCACTCGGAGTTGCCACCTCTGTGCCAATTG 1794    	1734    	1795 TGCTGAATCTATCACACTAGAAAAAACATTCTCAAGGTAAATGACTTGTGGACTATGTTCTGAATTG 1863    	1859  EcoRI   	1789    	1864 TCATTAAGTTTATTTCTGAAGTTAACGTTAACCTTCTGTTGAAATAATATCGTTCATAAAGATG 1932    	1904    
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FIGURE 10  
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1933 TCACGCCAGGACATGAGCTACACATCGCACATAGCATGAGATCAGGACGATTGTCACTCACTTCAA 2001  
 1971

SphI  
 NspI

2002 CACCTAAGAGCTTCTCTCACAGGCCACACATAATGCATGCAATATTACACGTGATCGCCATGCAA 2070  
 2015

SphI  
 NdeI  
 [AvaIII]  
 SspI

2037 2048 2053  
 2036 2044 2056

2071 ATCTCCATTCTCACCTATAATTAGGCCCTCGGCCTTCACTCTTACTCAAACAAACTCATCACTACA 2139  
 2099

SecI

2140 GAACATACACAAATGGCGAACAGCTCTCGCTCGGCCTCGCCTTGTTCTCCTTCACC 2208  
 METAlaAsnLysLeuPheLeuValSerAlaThrIleuAlaLeuPheLeuLeuThr  
 2171

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FIGURE 10  
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2209 AATGCCCTCCGCTACAGGACGGTTGAAAGATGATGCCACAAATCCAGCCGGCCATT AsnAlaSerValTyArgThrValValGluValAspGluAspAspAlaThrAsnProAlaGlyProHe 2220 2241 2242 2240	Sall HindII AccI           NaeI Cfr10I     Tth11II HindIII   NlaIV   ArgileProLysCysArgLysGluPheGlnGlnAlaGlnHisLeuLysAlaCysGlnTrpLysLys 2278 AGGATTCCAAATGTTAGGAAGGGAGTTTCAAGCAACACACCTGAAAGCTTGCACAAACAAATGGCTCCAC 2346 2325	Tth11II Tth11II   NlaIV   BbvII   LysGlnAlaMETGlnSerGlySerGlyProSerTrpThrLeuAspGlyGluPheAspPheGluAspAsp 2347 AAGCAGGCCAATGCAGTCCGGTAGTGGTCCAAAGCTGGACCCCTCGATGGTGAGTTTGATTGAAAGACGAC 2415 2363 2384	NlaIV   Apal   GsuI   HaeI   NspBII   SacI   Ksp632         ValGluAsnGlnGlnGlyProGlnGlnArgProProLeuLeuGlnGlnCysCysAsnGluLeuHis 2416 GTGGAGAACCAACAGGGCCCGCAGCAGGGCCACCGCTGCTGCCAGCAGTGGCTGCAACGAGCTCCAC 2484 2438 2444 2449 2455 2436	2269 2277 2267 2241 2242 2240 2342 2415 2481 2484
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FIGURE 10  
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2485	CAGGAAAGGCCACTTTGCGTTTGGCCCAACCTTGAAAGGCCATCCAAAGCCGTTAACAAACAGATTGCA GlnGluGluProLeuCysValCysProThrLeuLysGlyAlaSerLysAlaValLysGlnGlnIleArg	2553
2554	CAACAAACAGGGACAACAAATGCCAGGACAGGCAGATGCCAGAAAGTGAATTAGCCGTATCTACCAAGACCGCT GlnGlnGlnGlyGlnGlyGlnMETGlnGlnArgGlnValleSerArgIleTyrGlnThrAla	2622
	SecI	
	BbvII	
2623	ACGCACCTACCTAGAGCTTGCACACATCAGGCAAGTTAGCATTGCCCCCTTCCAGAACGACCATGCCCTGGG ThrHisLeuProArgAlaCysAsnIleArgGlnValSerIleCysProPheGlnLysThrMetProGly	2691
	2684	
	2687	
	SecI	
	DsaI	
2692	XbaI	2760
	AccI	
2692	CCGGGCTTCTACTAGATTCCAAACGAATAATCCTGGAGAGTGTGTATACCACCGGTGATATGAGTGTGGTT ProGlyPheTyr	2760
	2724	
2694	2736	
2692	2740	
	SecI	
	DsaI	
2761	XbaI	2829
	HpaI	
	HindII	
2774	GTTGATGTATGTAAACACTACATAGTCATGGTGTGTCCATAATAATGTAATAAGAAC	2774

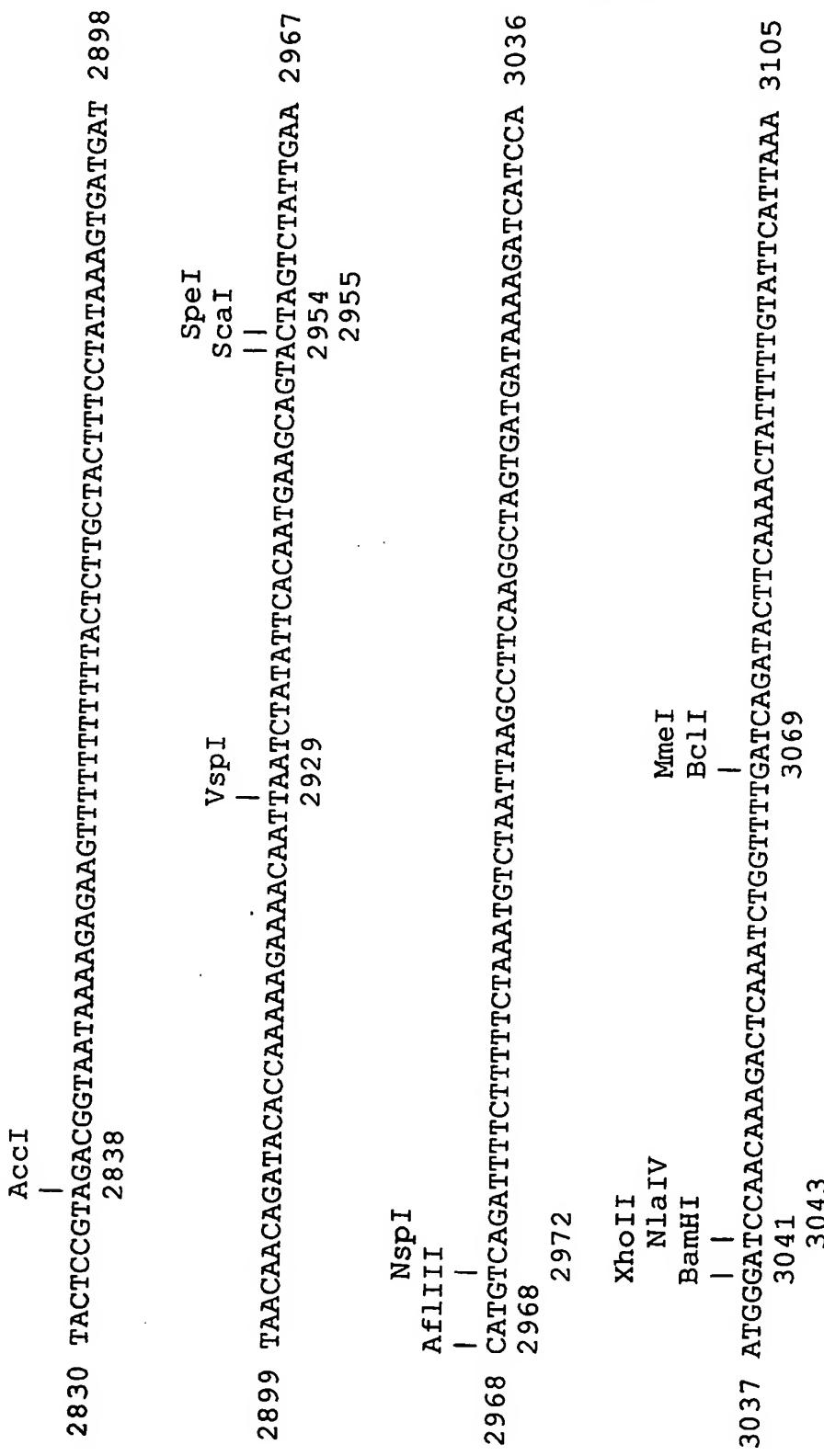


FIGURE 10  
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3106	TTATGCAAGTGTCTTTATTGGTGAAGGACTCTTTAGAAGCAAAGAACGACAAGCAGTAATAAAAAAA	Tth111
	3139	
	3174	
3175	ACAAAGTTCAAGTTAACGATTGGACTTATTGTCATTGAAATAATGATAGTATAGTATAATAATAAA	VspI
	3237	
3244	GTTTTATTATATAATGCTTGTCATTCAAGATTGAGAAACATTAAATATGATACTGTCCACATATCCAA	VspI
	3250	
3287		
3313	TATATTAAAGTTCAATTCTGTTCAAACATATGATAAGATGGTCAAATGATTATGAGTTTGTATTAC	NdeI
	3341	Tth111II
	3352	
3382	CTGAAGAAAAGATAAAGTGAGCTTGAGTTCTGAAAGGGTACGTGATCTTCATTCTGGCTAAAGCGA	Eco57I
	3404	
	3434	
3451	ATATGACATCACCTAGAGAAAGCCGATAAATAGTAAACTCTGTTCTGGTTTGGTTAATCAAACCGA	
	3519	

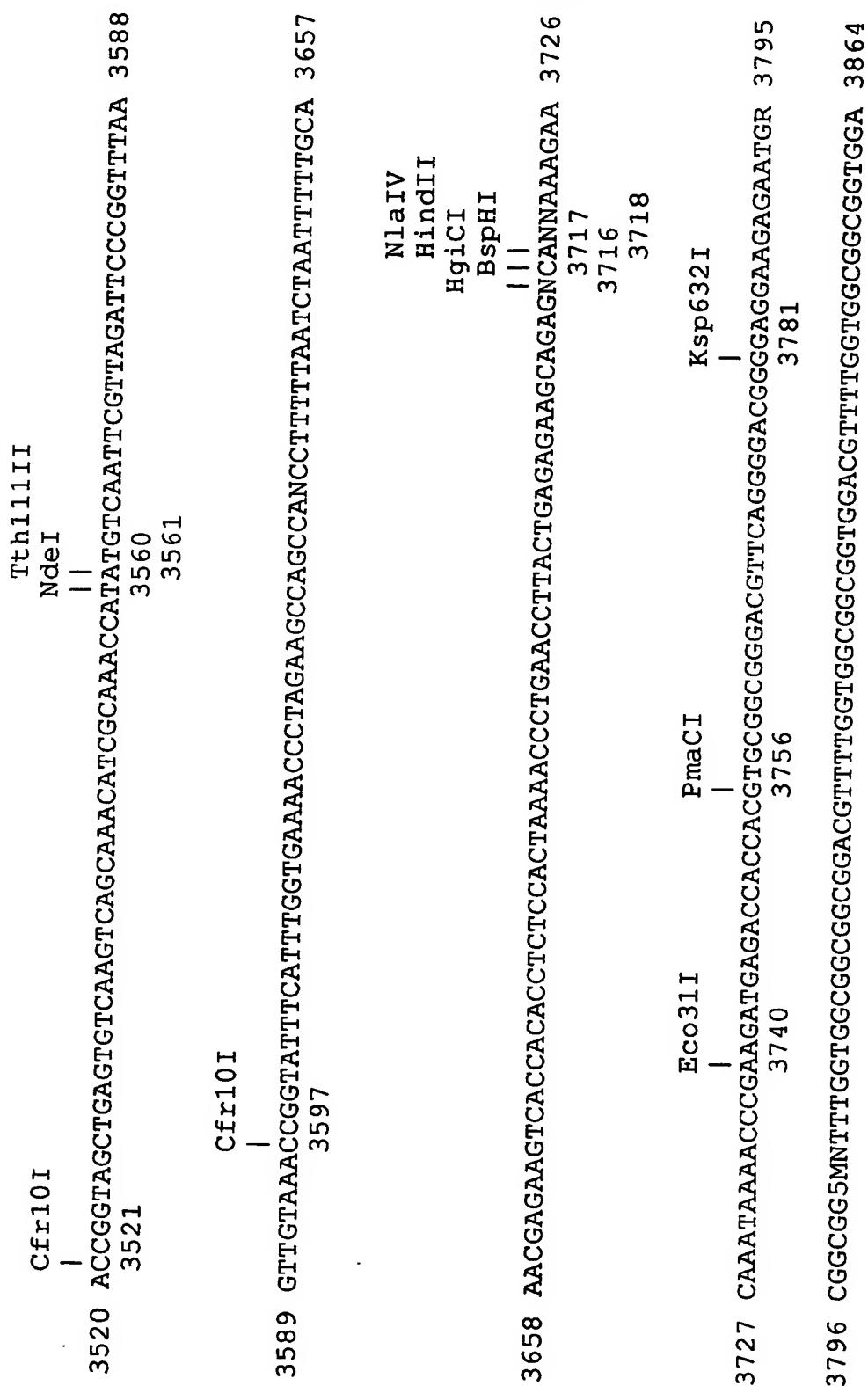


FIGURE 10  
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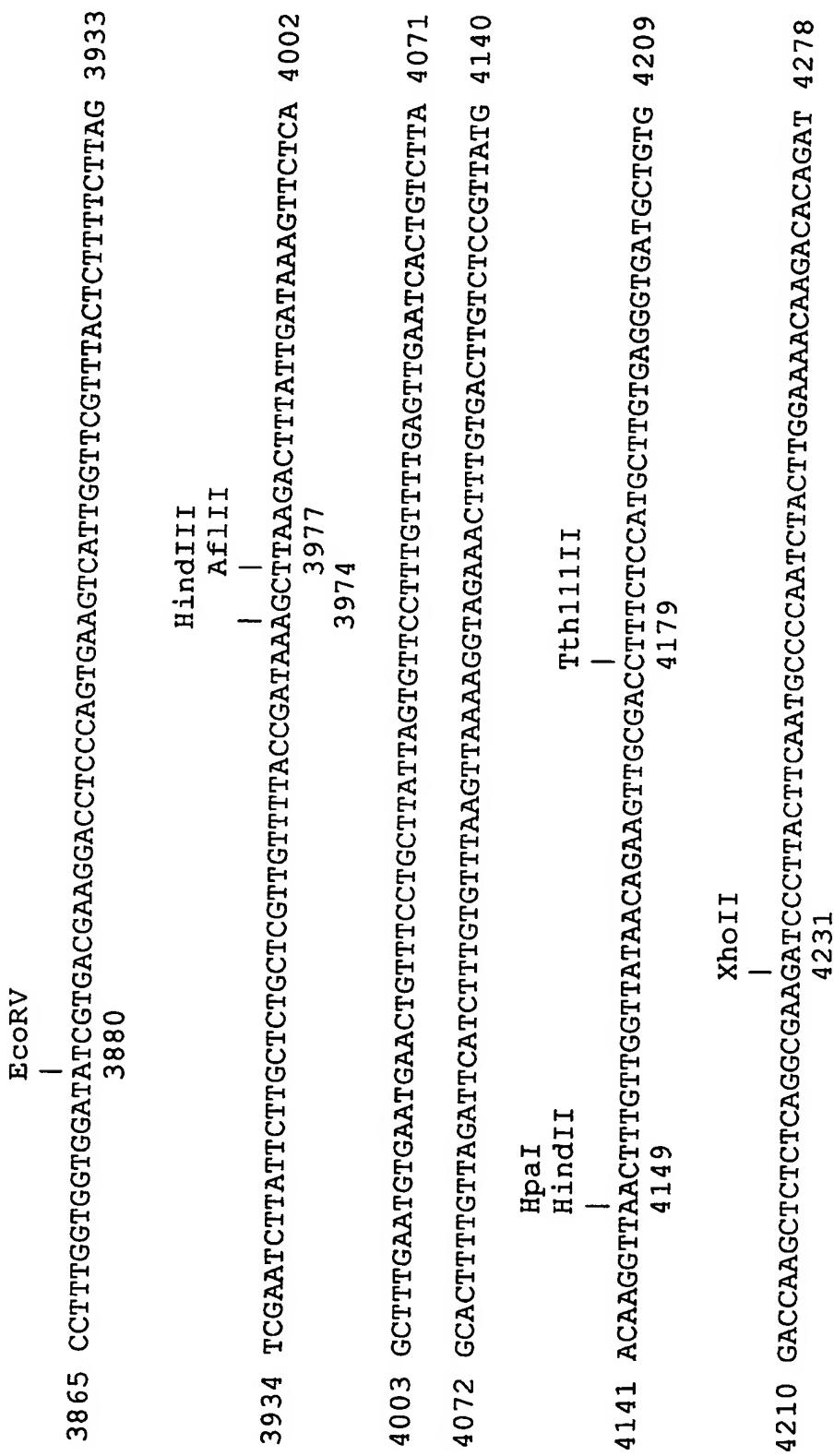
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		Sall	PstI	HindIII	HindII	
		XbaII	BspMI		AccI	ECORI
4279	GGGAAAGTTGAGATCCAAGGCTTGGGCTGCAGGTCGACGAATTG	4294	4302	4300	4316	4321
					4317	
						4313
						4315

FIGURE 10  
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# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/01746

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC IPC(5): C12N 1/21, 15/29, 15/82; C07H 15/12 U.S. CL.: 435/172.3, 240.4, 252.3; 536/27		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
U.S.	435/172.3, 240.4, 252.3 ; 536/27 800/205, DIG.69	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>		
USPTO AUTOMATED PATENT SYSTEM: DIALOG FILES BIOTECH AND PATENTS. SEE ATTACHMENT FOR SEARCH TERMS		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b> <sup>9</sup>		
Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
SEE ATTACHED PAGES		
<p>* Special categories of cited documents: <sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
24 June 1991		07 AUG 1991
International Searching Authority		Signature of Authorized Officer
RO/US		P. Rhodes <i>P. Rhodes</i>

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SEC. 3 SHEET) PCT/US91/01746

Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	World Soybean Research Conference III: Proceedings (Westview Press): Shibles (ed); Published 1985; Goodman et al; "Biotechnology and its impact on future improvements in soybean production and use"; pages 261-271. See pages 264-265.	1-22, 34-37
Y	Journal of Lipid Research; Volume 26; Issued 1985; Mattson et al; "Comparison of effects of dietary saturated, monounsaturated, and poly-unsaturated fatty acids on plasma and lipids and lipoprotein in man"; pages 194-202. See entire document.	1-22, 34-37
Y	EP A 0,255,377 (KRIDL et al) 03 February 1988. See entire document.	1-22, 34-37
Y	Trends in Biotechnology; Volume 5; Issued February 1987; Knauf; "The application of genetic engineering to oilseed crops"; pages 40-47. See entire document.	1-22, 34-37
Y	Trends in Biotechnology; Volume 7; Issued May 1989; Battey et al; "Genetic engineering for plant oils: potential and limitations"; pages 122-126. See entire document.	1-22, 34-37
Y	US, A, 4,446,235 (SEEBURG) 01 May 1984. See entire document.	1-22, 34-37
Y	US, A, 4,394,443 (WEISSMAN et al) 19 July 1983. See entire document.	1-22, 34-37
Y	Methods in Enzymology; Volume 71; Issued 1981; McKeon et al; "Stearoyl-acyl carrier protein desaturase from safflower seeds"; pages 275-281. See entire document.	1-22, 34-37
Y	Archives of Biochemistry and Biophysics; Volume 162; Issued 1974; Jaworski et al; "Fat metabolism in higher plants, properties of a soluble stearoyl-acyl carrier protein desaturase from maturing <i>Carthamus tinctorius</i> "; pages 158-165. See entire document.	1-22, 34-37
Y	The Journal of Biological Chemistry; Volume 257, Number 20; Issued 25 October 1982; McKeon et al; "Purification and characterization of the stearoyl-acyl carrier protein desaturase and the acyl-acyl carrier protein thioesterase from maturing seeds of safflower"; pages 12141-12147. See entire document.	1-22, 34-37

## "II. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SEC. I SHEET)

PCT/US01/01746

Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	Proceedings of the Flax Institute USA; Volume 41, Number 3; <sup>Issued 1971</sup> Downey et al; "Genetic control of fatty acid composition in oilseed crops"; pages 1-3. See entire document.	23-33, 38-45
X	EP, A,0323753 (WONG et al) 12 July 1989.	23-29, 31-33
Y	See entire document.	<u>38-39, 41-45</u> 30, 40
X	Journal of the American Oil Chemists Society; Volume 61, Number 1; Issued January 1984; Wilcox et al; "Genetic alteration of soybean oil composition by a chemical mutagen"; pages 97-100. See entire document.	23, 25-27, 29, <u>31, 33, 38-45</u> 30, 40
Y	Journal of the American Oil Chemists Society; Volume 59, Number 5; Issued May 1982; Wolf et al; "Effect of temperature on soybean seed constituents: oil, protein, moisture, fatty acids, amino acids and sugars"; pages 200-202. See entire document.	23, 25-27, 29, <u>31, 33, 38-45</u> 30, 40
Y	Lipids; Volume 4, Number 6; Issued 1969; Inkpen et al; "Desaturation of palmitate and stearate by cell-free fractions from soybean cotyledons"; pages 539-543. See entire document.	30, 40
Y	The Journal of Biological Chemistry; Volume 241; <sup>Issued April 1966</sup> Nagai et al; "Enzymatic desaturation of stearyl acyl carrier protein"; pages 1925-1927. See entire document.	30, 40
X	The Journal of Heredity; Volume 80, Number 3; Issued March 1989; Moore et al; "The inheritance of high oleic acid in peanut"; pages 252-253. See entire document.	23, 25-27, 29 <u>31, 33, 38-45</u> 30, 40
Y	Crop Science; Volume 24; Issued November-December 1984; Carver et al; "Developmental changes in acyl-composition of soybean seed selected for high oleic acid concentration"; pages 1016-1019. See entire document.	23, 25-27, 29 <u>31, 33, 38-45</u> 30, 40
X	Bodman et al., "Soybeans and Soybean Products: Processing of edible soybean oil" published 1951 by Interscience Publishers, Inc. (N.Y.), pages 649-725, see only pages 702-709.	<u>31 and 33</u> 32